

Exhibit 101

Reactive oxygen species in cancer

GEOU-YARH LIOU & PETER STORZ

Department of Cancer Biology, Mayo Clinic, 4500 San Pablo Road, Jacksonville FL 32224, USA

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Abstract

Elevated rates of reactive oxygen species (ROS) have been detected in almost all cancers, where they promote many aspects of tumour development and progression. However, tumour cells also express increased levels of antioxidant proteins to detoxify from ROS, suggesting that a delicate balance of intracellular ROS levels is required for cancer cell function. Further, the radical generated, the location of its generation, as well as the local concentration is important for the cellular functions of ROS in cancer. A challenge for novel therapeutic strategies will be the fine tuning of intracellular ROS signalling to effectively deprive cells from ROS-induced tumour promoting events, towards tipping the balance to ROS-induced apoptotic signalling. Alternatively, therapeutic antioxidants may prevent early events in tumour development, where ROS are important. However, to effectively target cancer cells specific ROS-sensing signalling pathways that mediate the diverse stress-regulated cellular functions need to be identified. This review discusses the generation of ROS within tumour cells, their detoxification, their cellular effects, as well as the major signalling cascades they utilize, but also provides an outlook on their modulation in therapeutics.

Keywords: Oxidative stress, reactive oxygen species, cancer, signal transduction

Abbreviations: 5-LOX, 5-Lipoxygenase; AP-1, activating protein-1; Ask-1, apoptosis signal-regulating kinase-1; BER, base excision repair; BITC, benzyl isothiocyanate; BPQ, benzo(a)pyrene quinines; CREB, cyclic AMP response element (CRE)-binding protein; CSC, cancer stem cell; ECM, extracellular matrix; EGCG, epigallocate-3-gallate; EGF, epidermal growth factor; EMT, epithelial-to-mesenchymal transition; Erk1/2, extracellular-regulated kinase 1/2; Ets, E twenty-six; FAK, focal adhesion kinase; FGF, fibroblast growth factor; GCS, glutamylcysteine synthetase; GPX, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulphide; GST, glutathione S-transferase; HIF-1, hypoxia inducible factor-1; ICAM-1, intracellular adhesion protein 1; IFN γ , interferon γ ; IKK, I κ B kinase; IL, interleukin; IOA, isoobtusilactone A; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MKP3, mitogen-activated protein kinase phosphatase 3; MMP, matrix metalloproteinase; NAC, N-acetyl-L-cysteine; NER, nuclear excision repair; NF- κ B, nuclear factor κ -B; NIK, NF- κ B-inducing kinase; PDGF, platelet-derived growth factor; PDK-1, 3'-phosphoinositide-dependent kinase-1; PDTC, pyrrolidine dithiocarbamate; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PKD, protein kinase D; Prx, peroxiredoxin; PST, pancratistatin; PTEN, phosphatase and tensin homologue deleted on chromosome 10; ROS, reactive oxygen species; SAL, salivine; SOD, superoxide dismutase; TGF β , transforming growth factor β ; TIMP, tissue inhibitor of metalloproteinase; TNF α , tumour necrosis factor α ; TPL, triphala; TRAF, TNF receptor-associated factor; VEGF, vesicular epithelial growth factor.

Reactive oxygen species

Reactive oxygen species are radicals, ions or molecules that have a single unpaired electron in their outermost shell of electrons. Due to this character, ROS are highly reactive. ROS can be categorized into two groups: free

oxygen radicals and non-radical ROS. Free oxygen radicals include superoxide ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), nitric oxide (NO^{\cdot}), organic radicals (R^{\cdot}), peroxy radicals (ROO^{\cdot}), alkoxy radicals (RO^{\cdot}), thiyl radicals (RS^{\cdot}), sulphonyl radicals (ROS^{\cdot}), thiyl peroxy

Correspondence: Peter Storz, Department of Cancer Biology, Mayo Clinic, Griffin Rm 306, 4500 San Pablo Road, Jacksonville FL 32224, USA. Tel: 904 953-6909. Fax: 904 953-0277. Email: storz.peter@mayo.edu

radicals (RSO_2^{\bullet}) and disulphides (RSSR). Non-radical ROS include hydrogen peroxide (H_2O_2), singlet oxygen ($^1\text{O}_2$), ozone/trioxygen (O_3), organic hydroperoxides (ROOH), hypochloride (HOCl), peroxynitrite (ONO^-), nitrosoperoxy carbonate anion ($\text{O}=\text{NOOCO}_2^-$), nitrocarbonate anion ($\text{O}_2\text{NOCO}_2^-$), dinitrogen dioxide (N_2O_2), nitronium (NO_2^+) and highly reactive lipid- or carbohydrate-derived carbonyl compounds. Among them, superoxide, hydrogen peroxide and hydroxyl radicals are the most well studied ROS in cancer.

Cellular sources for ROS

In cancer cells high levels of reactive oxygen species can result from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signalling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxygenases and thymidine phosphorylase or through cross-talk with infiltrating immune cells [1–3].

In mitochondria, ROS are produced as an inevitable byproduct of oxidative phosphorylation (Figure 1). The electron transport chain encompasses complexes

I–IV and ATP synthase on the mitochondrial inner membrane. Superoxide is generated at complexes I and III and released into the inter-membrane space (~80% of the generated superoxide) or the mitochondrial matrix (~20%) [4]. The mitochondrial permeability transition pore (MPTP) in the outer membrane of the mitochondrion allows the leakage of superoxide into the cytoplasm ([5] and [6] for a more detailed description of mitochondrial ROS generation). Superoxide is dismutated to H_2O_2 , either in the mitochondrial matrix (by MnSOD) or in the cytosol (by Cu/ZnSOD). H_2O_2 is a *bona fide* second messenger that is highly diffusible. Recent data suggest that hydrogen peroxide may cross cellular membranes through specific members of the aquaporin family [7]. For example, aquaporin-8 was detected in the inner mitochondrial membrane and suggested to function as a channel for water and potentially H_2O_2 [8]. In addition to the mitochondria, peroxisomes are other major sites of cellular ROS generation [9]. In these respiratory organelles, superoxide and H_2O_2 are generated through xanthine oxidase in the peroxisomal matrix and the peroxisomal membranes ([10,11], see [12] for a detailed review on ROS in peroxisomes).

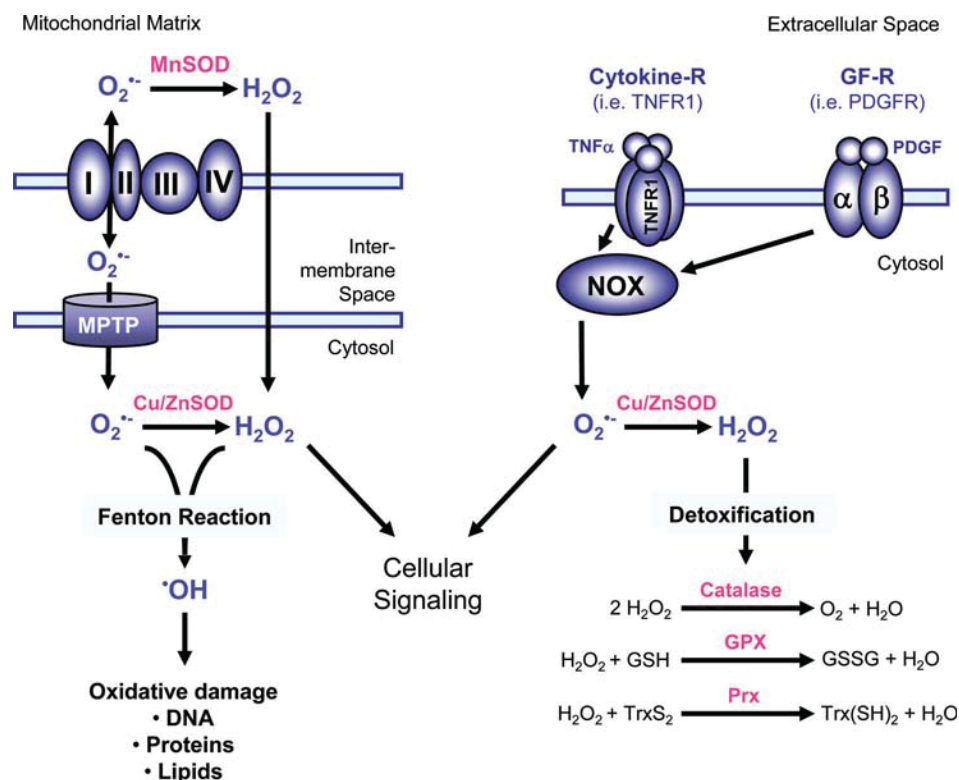


Figure 1. Major mechanisms of ROS generation and detoxification. Superoxide ($\text{O}_2^{\bullet-}$) radicals are generated at the inner membrane of the mitochondria as a byproduct of the electron transport chain and then release into the mitochondrial matrix or the cytosol via the mitochondrial permeability transition pore (MPTP). Superoxide is also generated through activation of NADPH oxidases (NOX), for example in response to growth factor receptor (GF-R) or cytokine receptor activation. SOD enzymes, such as MnSOD in the mitochondrial matrix or Cu/ZnSOD in the cytosol, reduce superoxide to H_2O_2 . Several cytosolic antioxidant systems, including catalase, glutathione peroxidase (GPX) and peroxiredoxins (Prx), detoxify cells from hydrogen peroxide by reducing it to water. Both hydrogen peroxide and superoxide contribute to cellular signalling but also can form hydroxyl radicals (•OH). Hydroxyl radicals are generated from $\text{O}_2^{\bullet-}$ and H_2O_2 in the Fenton reaction and have damaging functions for proteins, DNA and lipids.

Growth factors and cytokines stimulate the production of ROS to exert their diverse biological effects in cancer [13–16]. For example, an elevation of hydrogen peroxide and nitrite oxide levels was detected in tumour cells in response to interferon γ (IFN γ) and TNF α [17,18]. Further, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, transforming growth factor β (TGF β), interleukin-1 (IL-1), tumour necrosis factor α (TNF α), angiotensin and lysophosphatidic acid all induce the formation of superoxide [13,16,19–23]. Activation of the small GTPase K-ras downstream of growth factors or its oncogenic mutation has been tightly associated with increased generation of superoxide and the incidence of various cancers [24–26]. Dependent on the cellular system, growth factors and mutant K-ras elevate intracellular superoxide levels through NADPH oxidase or mitochondria [1]. NADPH oxidase can also be activated via the RhoGTPase Rac-1 [27]. Rac-1-mediated generation of superoxide is induced by cell surface receptors including c-Met [28]. Active Rac-1 further was implicated to induce 5-Lipoxygenase (5-LOX)-mediated generation of H₂O₂ [29].

Many cancers arise from sites of chronic irritation, infection or inflammation. Recent data have expanded the concept that inflammation is a critical component of tumour progression [30–32]. Macrophages induce the generation of ROS within tumour cells through secretion of various stimuli, such as TNF α [1]. Production of ROS by neutrophils and macrophages as a mechanism to kill tumour cells is well established. In these cells, a rapid burst of superoxide formation primarily mediated by NADPH oxidase leads to subsequent production of hydrogen peroxide [33,34]. Furthermore, during inflammation processes, activated macrophages also generate nitric oxide which reacts with superoxide to produce peroxynitrite radicals that are similar in their activity to hydroxyl radicals and contribute to tumour cell apoptosis [35].

Cellular detoxification from ROS

Under normal physiological conditions, the intracellular levels of ROS are steadily maintained to prevent cells from damage. Detoxification from ROS is facilitated by non-enzymatic molecules (i.e. glutathione, flavenoids and vitamins A, C and E) or through antioxidant enzymes which specifically scavenge different kinds of ROS (Figure 1).

Superoxide dismutases (SODs) are metalloenzymes which catalyse the dismutation of superoxide anion to oxygen and hydrogen peroxide. They ubiquitously exist in eukaryotes and prokaryotes. Superoxide dismutases utilize metal ions such as copper (Cu²⁺), zinc (Zn²⁺), manganese (Mn²⁺) or iron (Fe²⁺) as cofactors. The different SOD enzymes are located in different compartments of the cell and are highly specific in regulating linked biological processes [36].

Catalase facilitates the decomposition of hydrogen peroxide to water and oxygen. The major localization of catalase in most eukaryotes is in the cytosol and peroxisomes [37–39]. Peroxiredoxins are thioredoxin peroxidases that catalyse the reduction of hydrogen peroxide, organic hydroperoxides and peroxynitrite [40–42]. They are divided into three classes: typical 2-cysteine peroxiredoxins (PrxI–IV), atypical 2-cysteine peroxiredoxins (PrxV) and 1-cysteine peroxiredoxins (PrxVI). Interestingly, PrxI knockout mice show increased levels of oxidative stress and die prematurely of cancer [43]. The thioredoxin system consists of thioredoxin and thioredoxin reductase. The catalytic site of thioredoxin contains two neighbouring cysteines which are cycled between an active (reduced) dithiol form and an oxidized disulphide form [44]. In its active state, thioredoxin scavenges reactive oxygen species and keeps proteins in their reduced state [45]. Thioredoxin is regenerated by thioredoxin reductases which utilize NADPH as an electron donor [46].

The glutathione system includes glutathione (GSH), glutathione reductase, glutathione peroxidases (GPX) and glutathione S-transferases (GST). Glutathione protects cells from oxidative stress by reducing disulphide bonds of cytoplasmic proteins to cysteines. During this process, glutathione is oxidized to glutathione disulphide (GSSG). Glutathione peroxidases (GPX) catalyse the breakdown of hydrogen peroxide and organic hydroperoxides [47,48]. Glutathione reductase reduces GSSG and refills GSH pools [49]. Under physiological conditions, glutathione almost exclusively exists in its reduced form because of a constitutive activity of glutathione reductase in cells [50]. Glutathione S-transferases are detoxification enzymes that catalyse the conjugation of GSH to a variety of exogenous and endogenous electrophilic compounds [51–53]. GSTs are over-expressed in a wide variety of tumours to regulate MAPK pathways and are also involved in the development of resistance to chemotherapeutics [51].

Signalling pathways regulated by ROS in cancer

ROS-sensitive signalling pathways are persistently elevated in many types of cancers, where they participate in cell growth/proliferation, differentiation, protein synthesis, glucose metabolism, cell survival and inflammation [1]. Reactive oxygen species, particularly hydrogen peroxide, can act as second messengers in cellular signalling [16,54–57]. H₂O₂ regulates protein activity through reversible oxidation of its targets including protein tyrosine phosphatases, protein tyrosine kinases, receptor tyrosine kinases and transcription factors [1,27,58]. In the following paragraphs, we focus on ROS-mediated regulation of the mitogen-activated protein (MAP) kinase/Erk cascade,

phosphoinositide-3-kinase (PI3K)/Akt-regulated signalling cascades, as well as the I κ B kinase (IKK)/nuclear factor κ -B (NF- κ B)-activating pathways (Figure 2). Other ROS-regulated signalling pathways are included later.

ROS-mediated regulation of the MAPK/Erk1/2 pathway

The activation of the MAPK (mitogen-activated protein kinase)/Erk1/2 (extracellular-regulated kinase 1/2) pathway in cancer is mediated through growth factors and K-ras and was functionally linked to increased cell proliferation [59,60]. For instance, in human breast cancer cells, Erk1/2 activated by hydrogen peroxide generated as a byproduct during oestrogen metabolism increases cell proliferation [61,62]. Several mechanisms of how ROS activate Erk1/2 are known. For example Ras, which is an upstream activator for Erk1/2, can be activated directly through oxidative modification at its cysteine 118 residue, leading to the inhibition of GDP/GTP exchange [63]. ROS also activate upstream kinases of Erk1/2 such as p90^{RSK} [64,65]. It was recently shown that increased Erk1/2 activity in ovarian cancer cells in the presence of the high concentration of endogenous ROS results from sustained ubiquitination and loss of endogenous

MKP3 (mitogen-activated protein kinase phosphatase 3), a phosphatase that negatively-regulates Erk1/2 activity [64,65]. Additionally to its effects on cell proliferation, it was also shown in multiple cancers (i.e. ovarian cancer, breast cancer, melanoma and leukaemia) that the activation of Erk1/2 through ROS increases cell survival, anchorage-independent growth and motility [60,65,66].

While a role for ROS-activated Erk1/2 signalling in cell proliferation is well established [61,65,67], its ability to regulate cancer cell survival seems to be cell type specific [64,68,69]. For example, treatment of MCF-7 and MDA-MB-435 breast cancer cells with ROS scavengers or inhibitors that target Erk1/2 or its upstream kinase MEK (mitogen-activated protein kinase kinase) promote apoptosis and cell adhesion [70,71]. In an animal model for skin cancer, murine keratinocytes lacking Tiam1, an upstream activator of Erk1/2, show low levels of intracellular ROS [69]. These keratinocytes are more sensitized to apoptosis upon deprivation of EGF and insulin, implicating that Erk1/2 activation through Tiam1 and ROS is required for cell survival of skin cancer [69]. In contrast, in human pancreatic cancer and glioma cells, activation of Erk1/2 upon treatment with exogenous H₂O₂ triggers cell death and this probably is due to the high basal level of ROS in these cancer cells [72–76]. In line with

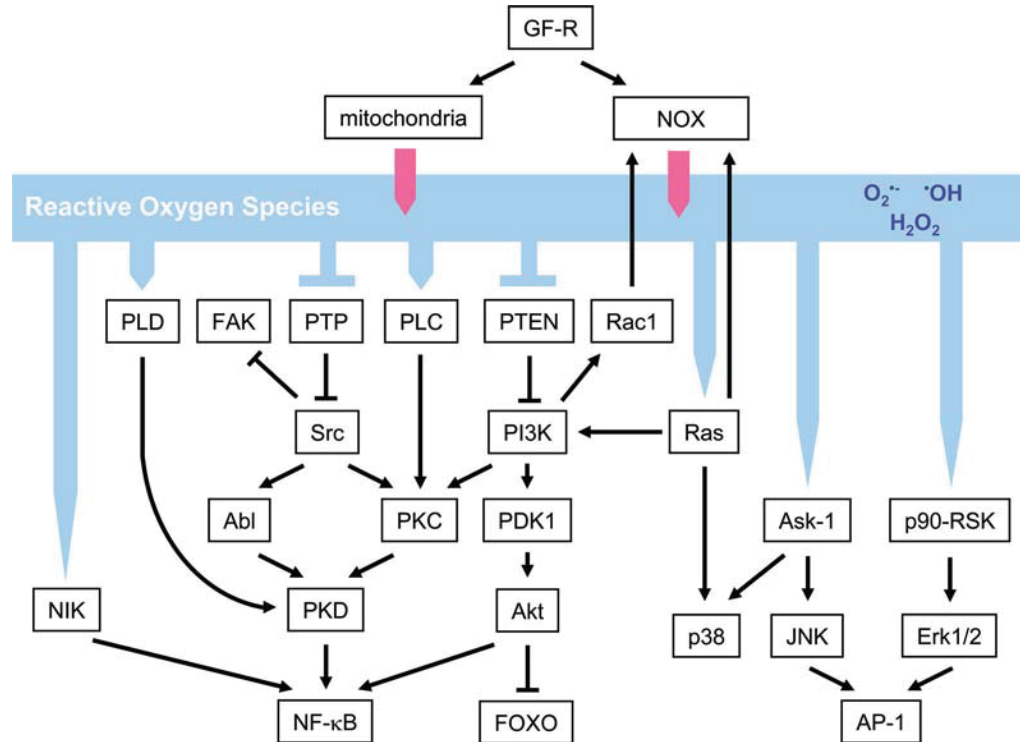


Figure 2. ROS-induced cellular signalling. Reactive oxygen species in cells can be generated by growth factor signalling through activation of the NADPH oxidase NOX1 or through the mitochondria. These ROS then can induce cellular signalling cascades by reversible oxidation of phosphatases such as PTEN or PTP in their active site cysteins or by direct oxidation of kinases such as Src. This leads to the activation of several signalling cascades such as a Src/PKD1-dependent NF- κ B activation mechanism, the MAPK (Erk1/2, p38 and JNK) signalling cascades, as well as the PI3K/Akt signalling pathway. Other mechanisms, by which ROS induce cellular signalling is through activation of redox-regulated transcription factors such as AP-1 or FOXO.

these *in vitro* data is an *in vivo* study showing that ROS-mediated increase of Erk1/2 activation loop phosphorylation suppresses the growth of pancreatic tumour cell xenografts [77].

Oxidative stress regulation of the PI3K/Akt pathway

Akt (or protein kinase B; PKB) mediates cell survival through phosphorylation and inactivation of its substrates such as the pro-apoptotic proteins Bad, Bax, Bim or FOXO transcription factors [78–83]. In breast cancer, ROS generation during oestrogen metabolism or other potential mammary carcinogens was shown to activate the PI3K/Akt signalling pathway [84,85]. Hydrogen peroxide generated by epithelial growth factor (EGF) in human ovarian cancer cells activates Akt and p70 S6K1, a substrate of Akt that regulates protein synthesis [86]. Moreover, the inhibition of ROS in the human pancreatic tumour cell line Panc-1 reduced the levels of phosphorylated (active) Akt and induced apoptosis [87]. Akt activity is tightly controlled by a signalling cascade that encompasses the kinases PDK-1 (3'-phosphoinositide-dependent kinase-1), mTOR and PI3K as well as the phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10). PDK-1 and mTOR regulate Akt activating phosphorylations at S473 and T308, whereas PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP₃), which serves as a membrane anchor [88]. PTEN negatively regulates PIP₃ levels and thus decreases Akt activity [89,90]. Treating cells with exogenous hydrogen peroxide it was shown that Akt and PDK-1 can be activated by oxidative stress [91,92]. This correlates with the observation that PTEN is reversibly inactivated by H₂O₂ [93]. Loss of PTEN increases basal levels of hydrogen peroxide and superoxide due to depletion of the expression of several antioxidant enzymes including peroxiredoxins and copper/zinc superoxide dismutase [94]. This suggests a constant activation of Akt through enhanced ROS production due to PTEN ablation, but also oxidative stress-mediated activation of its upstream kinases.

ROS regulation of the IKK/NF-κB pathway

In many cancers the transcription factor NF-κB is uncoupled from its normal modes of regulation and shows increased activity [95–98]. Recent studies have established a crucial role for NF-κB in tumour cell survival, regulation of cell cycle and proliferation, cellular adhesion and development of drug resistance in cancer cells during therapy [99–101].

NF-κB is a redox-regulated sensor for oxidative stress [102] and is activated by low doses of hydrogen peroxide [103]. When inactive, NF-κB is tightly bound to its inhibitor IκB that sequesters the transcription factor in the cytosol [104–108]. The canonical activation

of NF-κB is mediated through the NF-κB-inducing kinase (NIK) and the IκB kinase (IKK) complex, consisting of IKKα, IKKβ and NEMO. Upon its activation through cytokines such as TNFα or IL-1, NIK phosphorylates and activates its downstream targets, the kinases IKKα and IKKβ [104,109–111]. Active IKKs phosphorylate IκB and this leads to its subsequent ubiquitination and proteosomal degradation [112,113]. Degradation of IκB translocates NF-κB to the nucleus, where it acts as a transcription factor to induce the expression of anti-apoptotic and anti-inflammatory genes [114].

Oxidative stress activates NF-κB through a variety of distinct signalling pathways [115]. For example, treatment of MCF-7 breast cancer cells with TNFα, IL-1β or the mammary carcinogen sodium arsenite generates hydrogen peroxide and superoxide, which translates to the activation of NF-κB and increased cell proliferation [116–118]. In oral squamous carcinoma cells silencing of the antioxidant superoxide dismutase (SOD) increased basal ROS levels correlating with increased NIK and NF-κB activity [119]. The mechanism of how ROS activates NIK is most likely via oxidative inhibition of regulatory phosphatases [116]. Recent work from our group delineated an IKK-dependent NF-κB-inducing signalling pathway that is activated by increased cellular oxidative stress, induced either by exogenous treatment of cells with hydrogen peroxide, by rotenone-mediated mitochondrial generation of superoxide or inhibition of intracellular antioxidant systems such as the glutathione system [120,121]. In this pathway, NF-κB is activated through the lipase PLD1 and the kinases Src, Abl and Protein Kinase Cδ (PKCδ), whose signalling converge at the level of Protein Kinase D1 (PKD1) [120,122–124]. PKD1 is upstream of the IKK complex and mediates the activation of NF-κB through IKKβ [121]. In addition to this, IKK-independent activation of NF-κB in response to ROS can occur through tyrosine phosphorylation of IκBα, leading to a release from the IKK complex, but not to its degradation [125,126].

Specific functions of ROS in cancer

Oxidative stress-mediated signalling events have been reported to affect all characters of cancer cell behaviour [1,2,127]. For instance, ROS in cancer are involved in cell cycle progression and proliferation, cell survival and apoptosis, energy metabolism, cell morphology, cell–cell adhesion, cell motility, angiogenesis and maintenance of tumour stemness (Figure 3).

ROS in tumour cell proliferation

Low doses of hydrogen peroxide and superoxide stimulate cell proliferation in a wide variety of cancer cell

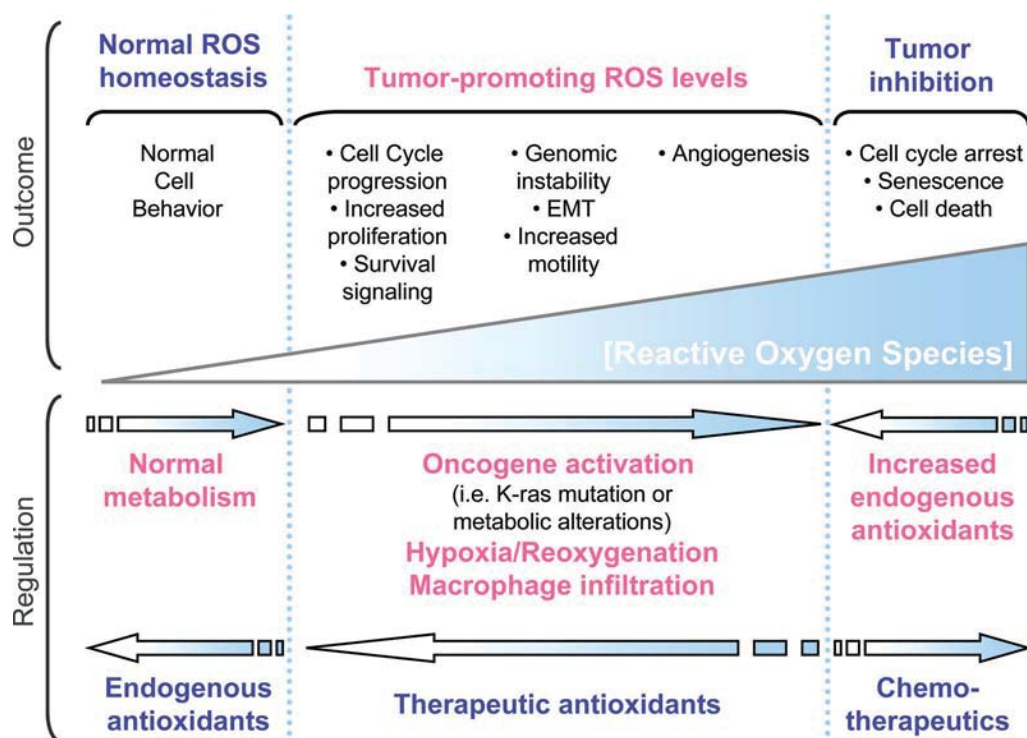


Figure 3. Generation, regulation and effects of cellular ROS. ROS are generated in normal cellular processes and cells express antioxidants to deplete intracellular levels of oxygen radicals. Tumourigenic events including oncogene activation (i.e. mutation of K-ras), metabolic alterations or macrophage infiltration or hypoxia/reoxygenation processes in tissues can increase intracellular ROS levels and promote tumour formation or progression. These tumour-promoting ROS levels can lead to cell cycle progression, increased proliferation and survival signalling, EMT, increased motility, genomic instability and increased angiogenesis and may be negatively-regulated by therapeutic antioxidants. Finally, excessive increase in intracellular ROS levels as mediated by chemotherapeutics, can induce cell cycle arrest, senescence or cell death of tumour cells, but may be repulsed by the tumour cells through an increase in the expression of endogenous antioxidants.

types [1,128]. For example, intracellular oxidative stress in breast cancer cells is increased through the translocation of oestrogen to the mitochondria [62, 129–131]. Mitochondria-derived ROS regulate both cell proliferation and quiescence. This is mediated by MnSOD activity which serves as a mitochondrial ROS switch [132]. Decreased MnSOD activity favours proliferation, due to increased superoxide and low hydrogen peroxide levels, while increasing MnSOD activity drives the proliferating cells to transit into quiescence, due to increased generation of hydrogen peroxide [133]. In breast cancer cells, inhibition of the mitochondrial uniporter blocks ROS generation and suppresses oestrogen-induced cell proliferation, suggesting a role of mitochondrial ROS in tumour growth [134]. Oestrogen-induced cell proliferation results from ROS-mediated activation of the Erk1/2 MAPK signalling pathway and the transcription factor CREB (cyclic AMP response element (CRE)-binding protein) [61,131].

Reactive oxygen species can upregulate the mRNA levels of cyclins that participate in the cell cycle to expedite G1 to S phase transition, including cyclin B2, cyclin D3, cyclin E1 and cyclin E2 [130]. It was shown that loss of the redox control of the cell cycle in normal MCF-10A cells may contribute to aberrant

proliferation [135]. The treatment of MCF-10A cells with the antioxidant NAC caused delays in the progression from G1 to S accompanied with a decrease in cyclin D1 levels [135]. Further, the environmental carcinogen sodium arsenite stimulates ROS production in breast cancer cells and potentiates S phase progression and subsequent cell proliferation [118]. Likewise, benzo(a)pyrene quinines (BPQs) imitate growth factor signalling and increase mammary epithelial cell growth rates through induction of superoxide and hydrogen peroxide [84].

Conversely, antioxidants inhibit tumour cell proliferation [136]. For example, pancreatic cancer cell lines generally show high basal levels of endogenous oxidative stress as compared to normal cells [1]. These increased ROS levels have been linked to increased proliferation. A stable ectopic expression of the highly-active antioxidant enzyme MnSOD reduces the cell growth rate of pancreatic tumour cells [72]. Moreover, the expression levels and activities of endogenous MnSOD, Cu/ZnSOD, catalase and glutathione peroxidase reversely correlate with cell doubling times in various pancreatic cancer cell lines [72,73]. ATM (ataxia telangiectasia mutated) is one of the proteins involved in cell cycle regulation that are activated by ROS. Patients lacking ATM show higher levels of oxidative

damage and similar effects, obtained with ATM knock-out mice can be rescued with administration of anti-oxidants [137,138]. Altogether, this suggests ROS as positive regulators of tumour cell proliferation by modulating key proteins in cell cycle progression.

ROS in apoptosis and cell survival

A disproportional increase in intracellular ROS can induce cancer cell cycle arrest, senescence and apoptosis. This can be achieved with cancer chemotherapy, depletion of cells from antioxidant proteins or generation of ROS by immune cells. Apoptosis is linked to an increase in mitochondrial oxidative stress that causes cytochrome *c* release, an unrevocable event that leads to the activation of caspases and cell death [139,140]. Additionally, superoxide generation through the Rac-1/NADPH oxidase pathway can also induce pro-apoptotic signalling [141].

Mitochondrial release of H₂O₂ and NO upon apoptotic signals leads to the activation of c-Jun N-terminal kinases (JNKs) [139,142]. In response to ROS, JNKs catalyse the phosphorylation and down-regulation of anti-apoptotic proteins such as Bcl-2 and Bcl-XL [139]. Both Bcl-2 and Bcl-XL have been shown to antagonize ROS generation and to protect cells from ROS-mediated apoptosis [143,144]. JNK also alters the composition of the Bax/Bcl-2 complex by increasing the expression of Bax, leading to formation of Bax homodimers, resulting in dissipation of mitochondrial membrane integrity [145–148].

p38, another MAPK family member, was also implicated in apoptotic signalling in response to increased generation of ROS. Both p38 and JNK are activated through Ask-1 (apoptosis signal-regulating kinase-1), whose activity is regulated by its interaction with thioredoxin. Thioredoxin is a redox-regulated protein that in its reduced form binds and inhibits Ask-1 [149, 150]. In addition to Ask-1-induced signalling cascades, other signalling proteins such as forkhead transcription factors (i.e. FOXO3a), p66Shc and p53 have been implicated in the induction of apoptosis in response to ROS [78,151]. For example, an interesting hypothesis is that constitutive oxidative stress in tumour cells may lead to the selection of p53-deficient clones that are resistant to apoptosis [1].

Death receptors such as the TNF receptor I mainly induce ROS generation via the mitochondria, leading to caspase activation and cell death [152]. However, TRAF4 (TNF receptor-associated factor4), a component of the TNF α signalling pathway, also binds to the NADPH oxidase complex to activate JNK [153], suggesting that death receptors may use several ways to induce ROS within cells. Notably, TNF-induced oxidative stress also mediates anti-apoptotic signalling by inducing the expression of MnSOD and catalase through NF- κ B [154].

In the above signalling events high levels of ROS turn on cell death signalling. However, it recently became clear that low levels of oxidative stress can also actively promote cell survival signalling. Such a ROS-mediated survival pathway is regulated by protein kinase D1 (PKD1) [120,121,124,155–157]. Elevation of intracellular mitochondrial ROS levels activates PKD1 and subsequently NF- κ B, leading to upregulation of antioxidant proteins such as MnSOD and anti-apoptotic proteins such as A20 and cIAPs [158]. In this pathway PKD1 is activated through the tyrosine kinase Src. Src directly phosphorylates PKD1, but also facilitates further activating phosphorylations through the kinases PKC δ (a member of the novel PKC family) and Abl [6,120,121,123,124,142]. The elimination of this pathway sensitizes tumour cells to oxidative stress and increases their susceptibility to ROS-mediated cell death [155–157,159,160].

Another anti-apoptotic protein that is activated by ROS in cancer is Akt, a serine/threonine kinase that fosters cell survival through phosphorylation and inactivation of its pro-apoptotic substrates [78–83]. Akt activity is induced by multiple receptor tyrosine kinases such as PDGF-R as well as constitutively-active K-ras via activation of PI3K.

ROS as regulators of cell motility and metastasis

The treatment of carcinoma cells with hydrogen peroxide prior to intravenous injection into mice enhanced metastasis [161]. Additionally, sub-populations of the low- or non-motile breast cancer cell line MCF-7 that possess higher levels of endogenous ROS than the parental cells showed increased motility, and orthotopic tumours generated with these cell lines metastasized to lung, liver and spleen [162]. Furthermore, metastatic breast cancer and highly-invasive pancreatic cancer cells show lower levels and activities of the antioxidant enzyme MnSOD [73,163,164]. This illustrates that the intracellular redox state governs crucial steps for the metastatic process. This comprises decreased cell adhesion to the extracellular matrix, anchorage-independent survival, increased migratory and invasive potential, as well as intravasation.

Cell adhesion and migration are dependent on integrin binding to the extracellular matrix. Integrins elevate oxidant levels mainly by increasing cyclooxygenase-2 [165], but also through 5-lipoxygenases (5-LOX) and mitochondria [27,166]. In this context, an increase in mitochondrial ROS was linked to a first cellular contact with ECM and increases in cytosolic ROS were shown to contribute to cytoskeleton remodelling and actin stress fibre formation during a later phase of the process [27,167]. Targets for mitochondrial ROS in these processes are SHP-2 and FAK (focal adhesion kinase), while cytosolic ROS target the phosphatases LMW-PTP and SHP-2, receptor tyrosine kinases, Src-family kinases, FAK

and structural proteins such as β -actin (in more detail reviewed in [27]). Activation of phosphatases and Src occurs through direct oxidation, whereas activation of FAK is probably indirect through upstream signalling events leading to its tyrosine phosphorylation [168]. Both Src and FAK are initiators of focal adhesion formation in adherent cells, contributing to cell spreading, cell migration and prevention of cell death by anoikis.

Non-transformed cells require an anchorage to extracellular matrix (ECM) to execute the mitotic programme. In this process ROS act as key second messengers to facilitate proper mitosis [27,169]. A synergistic signalling between growth factors (GF) and integrins leads to an oxidative burst through a Rac-1-dependent increase in mitochondrial ROS [13,170]. This leads to oxidative inhibition of PTPs, activation of Src and other protein tyrosine kinases or structural proteins, with the net effect of increasing cell adhesion to ECM, cell spreading and proliferation.

Loss of cell-to-matrix adhesion in non-transformed cells triggers anoikis, a specific type of apoptosis. In contrast to non-transformed cells, tumour cells are protected from this process and show increased cell proliferation and independence of anchorage. Such resistance to anoikis allows tumour cells to survive outside their 'normal' environment and to metastasize and form new colonies at distant sites. The mechanism of how tumour cells become independent of cell attachment signals is most likely through increased generation of intracellular ROS. Such increase in oxidative stress seems to mimic autocrine/adhesive signals, which in normal cells are mediated by growth factor and integrin signalling. For example, in prostate cancer cells redox-regulated anoikis resistance is mediated via Src and the EGF receptor [171]. Subsequently, this results in a constitutive deregulation of mitogenic pathways and proliferation independent of anchorage. It further allows cancer cells to abolish anoikis signals and escape apoptotic responses after a loss of cell/ECM contacts (for an excellent review on this topic see [27]).

Before cells migrate to distal sites, they undergo epithelial-mesenchymal transition (EMT) to release themselves from the restraint of the basal membrane. During this process, metalloproteinases (MMPs) are upregulated to degrade the proteins that compose the basal membrane. Treatment of murine mammary epithelial cells with MMP-3, a stromal protease that is upregulated in mammary tumours, increased their intracellular ROS levels (mainly H_2O_2) and led to EMT through induction of Rac1b RhoGTPase [172]. Moreover, application of NAC (*N*-acetyl-L-cysteine) to remove ROS abolished MMP-3-induced EMT [172], bolstering that MMP induces oxidative stress to lead to malignant transformation. This increase in ROS mediates oxidative damage to DNA and genomic instability. It further stimulates the expression of

Snail, which previously was identified as one of the key-transcription factors regulating EMT. Other ROS-regulated genes relevant to EMT are E-cadherin, integrins and MMPs [173].

Activation of Rac and subsequent generation of ROS leads to NF- κ B activation and MMP-1 production in response to integrin-mediated cell shape changes [170]. Rac-1 mediated changes in cellular ROS levels also increase the migratory potential of MCF-7 and T47D breast cancer cells, probably through NF- κ B [174]. Similarly, Rac-1 is a downstream target for c-Met and Rac-1-mediated ROS generation was involved in Met's prometastatic signalling [28]. Moreover, Rac-1 has important functions in ROS mediated actin reorganization of migrating tumour cells [175]. Multiple processes regulate actin reorganization at the leading edge of migrating cells including the actin-severing protein cofilin [176,177]. Rac-1 activates NADPH oxidase (NOX) and ROS generated by this enzyme have been shown to activate the cofilin pathway and thus contribute to increased cell migration [177,178]. The tyrosine kinase Src also regulates NADPH oxidase 1 (NOX1) induced generation of ROS [179]. NOX1 is capable of transforming cells and is also required to maintain the transformed state [87,174]. NOX1-mediated ROS generation has been shown to be necessary for the formation of invadopodia, actin cytoskeleton-based structures that tumour cells use to invade [180].

Matrix metalloproteinases facilitate the degradation and reorganization of the extracellular matrix and their increased activation was associated with primary tumour growth, angiogenesis, increased tumour cell invasion, blood vessel penetration and metastasis [181–184]. ROS regulate not only the expression of MMPs, but also the inactivation of their inhibitors TIMP (tissue inhibitor of metalloproteinase) [185,186]. An important step in oxidative stress-mediated expression of MMP genes is the dismutation of mitochondrially-generated superoxide to hydrogen peroxide [187]. Hydrogen peroxide then regulates the expression of MMPs through activation of the Ras-Erk1/2-Ets (E twenty-six), Rac-1-JNK-AP-1 (activating protein-1) or p38 signalling pathways [188] (for a review on this topic see [184]). Further, the redox-sensitive transcription factors NF- κ B and FOXO3a have been described as regulators of MMP expression [1,159]. Additionally to regulating MMP expression, ROS also can lead to the direct activation of MMPs through reactions with thiol groups in their catalytic domain [189].

Finally, ROS may also promote tumour cell metastasis by increasing the vascular permeability [181]. Increased activity of Rac-1 in primary endothelial cells mediates a loss of cell-cell adhesions and loosens the integrity of the endothelium, which allows the intravasation of cancer cells [190]. It was shown that reverse (basolateral-to-apical) transendothelial migration

(TEM) of human melanoma cells is induced by hydrogen peroxide and can be blocked by thioredoxin [191]. Oxidative stress also regulates the expression of interleukin-8 (IL-8) and the cell surface protein ICAM-1 (intracellular adhesion protein 1, CD54) through NF- κ B. Both ICAM-1 and IL-8 can regulate the trans-endothelial migration of tumour cells [192]. Further, phosphorylation of the heatshock protein Hsp27 by ROS-activated p38 induces changes in actin dynamics in vascular endothelial cells, which may contribute to facilitate invasive processes [193].

Hypoxia as a factor leading to tumour progression

Within a growing tumour mass cancer cells repeatedly face cycles of hypoxia and reoxygenation [194–196]. Limitations in oxygen supply due to prolonged hypoxia can result in cell death. Tumour cells can use the ‘Warburg effect’, a metabolic switch to glycolysis, to adapt to low oxygen tension [197]. Normal and tumour cells differ significantly in energy metabolism. Glucose is the primary energy source for normal cells. Normal cells switch to anaerobic glycolysis only when adequate oxygen supply is not available and mitochondrial function is suppressed [198]. A shift from aerobic to anaerobic metabolism in tumour cells occurs even under conditions of normoxia or after mitochondrial dysfunction, oncogenic transformation or loss of tumour suppressor genes [196,199].

The adaption of tumour cells to hypoxia contributes to the malignant phenotype and to aggressive tumour progression [200]. Hypoxia induces several transcription factors including HIF-1 (hypoxia inducible factor-1), which is composed of two sub-units HIF-1 α and HIF-1 β [196,200]. Under normal growth conditions HIF-1 is regulated by oxygen-dependent prolyl hydroxylases (PHDs) and the VHL ubiquitin ligase, which promote its proteosomal degradation [201]. However, HIF-1 becomes transcriptionally-active under low oxygen conditions. It was shown that under hypoxic conditions MnSOD suppresses the induction of HIF-1 α in human breast carcinoma cells. This suggests that superoxide may contribute to HIF-1 α accumulation [133]. However, increased generation of H₂O₂ also led to accumulation of HIF-1 α , suggesting that both types of ROS can increase HIF-1 α levels [133]. Increased HIF-1 α expression has been shown to correlate with poor prognosis and increased cancer cell invasiveness. HIF-1 regulates glycolysis-related genes and inhibits mitochondrial respiration (reviewed in [196]), resulting in hypoxic adaption of tumour cells. This leads to glycolytic ATP generation [202], reduced formation of mitochondrially-generated H₂O₂, enhanced survival of poorly oxygenated cells and regulation of EMT- and metastasis-related genes [203]. HIF-1 also prevents intracellular acidification, which leads to an increased formation of lactate and CO₂ [202], both

favouring extracellular matrix degradation and cell invasion [204].

Role of oxidative stress in angiogenesis

With increased tumour growth, more nascent blood vessels are developed to facilitate oxygen and nutrient supply to the centre of the tumour [205,206]. Several lines of evidence suggest a role for ROS in augmenting angiogenesis. For example, hypoxic conditions stimulate blood vessel development, whereby the blood flow in these new vessels is often chaotic, causing oxidative stress through periods of hypoxia and reoxygenation [181]. It was shown with a mouse model for breast cancer that administration of Mn(III) orthotetrakis-N-ethylpyridylporphyrin, a potent scavenger of reactive oxygen and nitrogen species, attenuates angiogenesis by modifying the density of microvessels and the proliferation rate of endothelial cells [207].

Angiogenesis is mediated through growth factors such as vesicular epithelial growth factor (VEGF) [208–210]. VEGF expression can be regulated by nutrient deprivation and hypoxia, which both increase intracellular levels of reactive oxygen species [211]. In such an environment HIF-1 and its co-factor p300 initiate gene expression including the expression of VEGF [212,213]. On the other hand, suppression of endogenous ROS by mitochondrial inhibitors or glutathione peroxidase decreases HIF-1 induction and VEGF expression in cancer cells [214]. Growth factor-mediated activation of Akt and subsequent formation of superoxide and H₂O₂ also lead to an induction of HIF-1 followed by expression of VEGF [86,215]. This is blocked when cells are pre-treated with catalase [86]. The knockdown of PTEN, a negative-regulatory phosphatase for the PI3K/Akt pathway, enhances VEGF secretion [216]. This is probably mediated by an increase in basal levels of hydrogen peroxide and superoxide, due to decreased expression of several antioxidant enzymes such as peroxiredoxins and Cu/ZnSOD [94].

ROS-induced secretion of matrix metalloproteinases such as MMP-1 from tumour cells promotes vessel growth within the tumour microenvironment. Further, a transient expression of MMP-1, MMP-2 and MMP-9 correlates with an increase in ROS during formation of capillary-like structures, implicating that MMP-mediated angiogenesis also occurs through upregulation of ROS [217]. ROS can also trigger vasodilation to increase the blood supply of tumours through activation of heme oxygenase-1, a enzyme that generates carbon monoxide or induces the formation of nitric oxide [218].

ROS and redox regulation in cancer stem cells

It is well established that after chemo- or radiotherapy a small sub-population of surviving primary cancer cells

can initiate recurrence. This sub-population of cells, termed cancer stem cells (CSC), expresses stem cell markers and is highly drug resistant. CSCs utilize redox-regulatory mechanisms to promote cell survival and tolerance to treatment [219,220]. As previously discussed, the accumulation of ROS is thought to contribute to the conversion of normal cells to cancer cells by mediating genomic instability, oncogenic growth, ECM independency and increased motility. In contrast to cancer cells, which maintain these high ROS levels during all stages of malignancy, cancer stem cells have an increased antioxidant capacity [221]. Keeping endogenous and induced ROS at moderate levels mediates drug resistance and allows these cells to survive during treatment, resulting in both stemness and cancer-initiating capabilities. Diehn et al. [222] recently showed that human and murine mammary epithelial cancer stem cells contain lower concentrations of ROS, specifically superoxide, than the more mature progeny, but also normal epithelial cells. They further demonstrated that these differences in ROS levels are critical for maintaining stem cell function. When compared to their normal tumour cell counterparts, CSCs showed increased expression of a variety of enzymes that contribute to oxygen radical scavenging [222]. Particularly genes regulating or involved in glutathione synthesis, including glutathione synthetases and glutamate cysteine ligase, were increased in their expression. Also increased was the expression of FOXO1, a forkhead transcription factor that was previously implicated in the regulation of other ROS scavengers such as SOD and catalase to confer resistance to oxidative stress in haematopoietic stem cells [223].

Since ROS are critical mediators of ionizing radiation-induced therapy [224,225] the expression of antioxidants in CSCs prevented DNA damage and protected cells from irradiation-induced cell death [222]. L-S,R-buthionine sulfoximine (BSO)-mediated pharmacological depletion of the ROS scavenger GSH in epithelial CSC markedly decreased their clonogenicity and resulted in increased radiosensitization [222]. Consequently, CSC-enriched populations accumulated fewer single and double strand breaks in their DNA after irradiation. Due to high levels of antioxidant signalling, cancer stem cells may also not be responsive to other (chemotherapeutic) treatments that target cancer cells by increasing intracellular ROS levels. To reduce recurrence in response to conventional therapy cancer stem cells have to be additionally targeted under consideration of their unique redox status. It will be interesting to see if decreasing oxidative defenses in cancer stem cells *in vivo* will cause them to lose their stemness, and if a combination therapy with standard chemotherapy is effective to eliminate both tumour and cancer stem cells.

Random damaging functions of ROS

Increased levels of reactive oxygen species can lead to 'non-specific' damage of macromolecules such as

DNA, proteins and lipids. Some ROS such as H_2O_2 are not very reactive towards DNA and most of the damaging effects on DNA are due to hydroxyl ions, which are generated via the Fenton reaction [226]. In this reaction transition metals such as iron and copper donate or accept free electrons during intracellular reactions and use H_2O_2 to catalyse free radical formation. Hydroxyl radicals attack DNA rapidly due to their high diffusibility, which results in formation of DNA lesions including oxidized DNA bases, single strand and double strand breaks [227,228]. DNA adducts are removed by either the base excision repair (BER) or the nuclear excision repair (NER) pathways [229]. Cells incapable to completely repair DNA lesions (i.e. due to deficient DNA repair enzymes) undergo apoptosis to ensure these mutations will not be passed on to progeny cells. However, under certain circumstances, the cells harbouring DNA mutations successfully escape programmed cell death, which raises a high chance for cancerous growth.

The oxidative modification of proteins by reactive species is implicated in the aetiology or progression of various disorders and diseases. The major damage of ROS to proteins is modification in their amino acid residues, resulting in altered functions. Some ROS-induced modifications also increase protein carbonylation, nitration of tyrosine and phenylalanine residues, protein degradation [230] or lead to formation of cross-linked and glycated proteins [231,232]. The oxidized amino acid residues of proteins can influence their ability in signal transduction mechanisms. For example, irreversible oxidation of phosphatases within the catalytic sites hinders their enzymatic activity [233]. Oxidative alterations of enzymes also impact DNA repair efficiency, the fidelity of DNA polymerase during replication/synthesis and transcriptional activity, which tightly associates with cancer onset [1,234–236].

Other cellular targets of ROS are lipids. ROS react with polyunsaturated or polydesaturated fatty acids to initiate lipid peroxidation [237,238]. Lipid oxidation generates numerous genotoxic molecules such as malondialdehyde, 2-alkenals and 4-hydroxy-2-alkenals [239, 240]. ROS-induced lipid peroxidation can be used as a tumour marker, as shown in clinical studies [241]. For example, the detection of thiobarbituric acid-reactive substances in the serum of patients with colorectal cancer indicates a high level of lipid peroxidation.

Application of ROS and antioxidants in cancer therapy and prevention

Many chemotherapeutic strategies are designed to exuberantly-increase cellular ROS levels with the goal to induce irreparable damages, subsequently resulting in tumour cell apoptosis (for a detailed review on the use of ROS in cancer therapy see [221]). Dependent on the tumour type, this can be achieved through chemotherapy or radiation therapy [1,242–244]. For example,

for pancreatic cancer, to date only few treatment strategies have been proven as effective for therapy and these include combination therapy of gemcitabine with trichostatin A, epigallocate-3-gallate (EGCG), capsaicin and benzyl isothiocyanate (BITC) [148,245–249]. All of these drugs share the same mechanism, namely to elevate intracellular ROS levels to trigger apoptosis [146,148,250,251]. Another compound that modulates ROS levels and is currently tested for its potential use in tumour therapy is Sulindac, a FDA-approved, non-steroidal and anti-inflammatory drug. Sulindac enhances intracellular ROS levels and renders colon and lung cancer cells more sensitive to H_2O_2 -induced apoptosis [252]. In addition, Aminoflavone (5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methylchromen-4-one; AF) induces cell death in MCF-7 and MDA-MB-468 breast cancer cells, but is not toxic for non-malignant MCF-10A breast epithelial cells [253,254]. Upon treatment with Aminoflavone, an increase of intracellular ROS is detected, correlating with increased activation of Caspase 3 and subsequent apoptosis. The inhibition of ROS generation by pre-treatment of cells with N-acetyl-L-cysteine (NAC) reverses Aminoflavone-induced cell death [254]. Several compounds such as IOA, pancratistatin (PST) and triphala (TPL) induce apoptosis of breast cancer cells through similar mechanisms as Aminoflavone, which is to increase intracellular ROS levels through dissipation of the mitochondrial membrane potential [255–260].

Mitochondrial DNA codes for several respiratory chain sub-units and is more vulnerable to DNA damage than nuclear DNA. The exposure of cells to ionizing radiation can lead to mitochondrial complex II dysfunction and increase the steady state levels of reactive oxygen species and contribute to genomic instability [261]. In human cancer, mutations in mitochondrial genes, such as the gene encoding cytochrome *c* oxidase II, are associated with increased ROS generation [262]. However, the susceptibility of mitochondrial DNA to ROS-induced mutation may also be utilized for therapy. For example, chemotherapeutic treatment of cancer patients with DNA damaging agents can lead to cell death by inducing mutations in the mitochondrial DNA that increase cellular ROS to a toxic level [262].

As discussed above, when compared to normal cells, cancer cells show increased sensitivity to glucose-induced cytotoxicity and it was suggested that increased glucose metabolism in cancer cells can compensate excess metabolic production of ROS. For example, glucose metabolism inhibits apoptosis in cancer cells through redox inactivation of cytochrome *c* [263]. Therefore, it was concluded that inhibition of glucose metabolism may provide a target for selectively targeting cancer cells by enhancing their oxidative stress levels to promote cell death [264]. 2-deoxyglucose (2DG), a glucose analogue that can not be metabolized, increased

oxidative stress levels and caused cell death in pancreatic and prostate cancer cells [265,266]. Moreover, this can be enhanced by additionally increasing cellular ROS levels with mitochondrial electron chain blockers [267].

Modulation of intracellular ROS levels can also be utilized to target oxidative stress-mediated tumour progression. For example, a loss of cell adhesion in tumour cells and anchorage-independent survival is tightly linked to a gain of cell motility and increased invasiveness. Salvicine (SAL) is a compound originally identified as a topoisomerase II poison and has been entered in a Phase II clinical trial for cancer therapy. Treatment of invasive MDA-MB-435 breast cancer cells with SAL causes rounded cell morphology, which indicates a decrease in cell adhesion [71]. The inhibition of ROS by the free radical scavenger NAC restores cell adhesion of MDA-MB-435 cells, suggesting that ROS augment their metastatic ability.

Since evidence from clinical and bench studies indicate that elevated intracellular ROS contribute to early events involved in cancer initiation and progression, an opposite approach to mediating an increase in cellular ROS levels is to use antioxidants to deplete tumour cells from ROS-induced survival signalling pathways. Such treatment may also have preventive functions. For instance, clinical studies have linked gain of oncogenic mutations in K-ras and subsequent ROS formation or pancreatic inflammation (pancreatitis) and macrophage-mediated generation of hydrogen peroxide and superoxide to events leading to an increased risk for pancreatic cancer [268–270]. Other examples are individuals with a high cancer risk due to the deficiency of inherited tumour suppressor genes such as p53 or PTEN. For these groups a treatment with antioxidants may be effective in delaying or even preventing tumour development. Depending on the therapeutic strategy, a use of antioxidants in combination therapy may have an adverse effect on anti-cancer drugs that act on tumor cells by increasing ROS levels to induce cell death. However, a combination therapy with antioxidants and therapeutics that induce apoptosis independent of oxidative stress may be effective. Antioxidants under development for clinical use are for example the SOD mimetic EUK-134 [271] or a mimetic of glutathione disulphide named NOV-002 [272].

In conclusion, to tailor specific combination therapy and to decide which strategy to use, chemotherapeutics that excessively increase intracellular ROS to reach a toxic level or antioxidants may be dependent on the tumour type and stage, the type and level of endogenous ROS as well as abundance of ROS-induced survival pathways.

Summary

After malignant transformation many cancer cells show a sustained increase in intrinsic generation of

reactive oxygen species, which maintains the oncogenic phenotype and drives tumour progression. Redox adaption through upregulation of anti-apoptotic and antioxidant molecules allows cancer cells to promote survival and to develop resistance to anti-cancer drugs. Little is known about how an increase in intracellular oxidative stress levels is sensed and transduced into ROS-induced specific intracellular signalling to regulate the expression of antioxidant and survival genes [142]. The dependence of tumour cells and cancer stem cells on their antioxidant capacity makes them vulnerable to agents that dampen antioxidant systems. There is a realistic prospect for treatments aimed to dramatically increase intracellular ROS to kill cancer cells by decreasing their antioxidant capacity [1]. This may be obtained using compounds that inhibit antioxidant systems or through inhibition of specific signalling pathways that upregulate antioxidants in cancer cells. The resulting increase in reactive oxygen species then may induce tumour cell death either through random damaging functions of ROS or by specific induction of apoptosis via death signalling pathways. The advantage of such a strategy is that normal cells are not significantly affected since they have lower basal ROS levels and therefore are less dependent on antioxidants. However, it is possible that a threshold of toxicity in these cancer cells is not reached and that the additional increase in ROS further causes more mutations or drives cell migration and invasion [221,273]. Therefore, a combination of inhibitors of antioxidant systems with pharmacological agents with pro-oxidant properties to increase ROS levels within tumour cells may be needed to overwhelm antioxidant systems over the threshold of toxicity [1,221]. It becomes evident that a much more detailed understanding of ROS-mediated signalling in tumour cells is necessary to develop new strategies for such a redox modulation-based therapeutic intervention to selectively kill cancer cells and overcome drug resistance.

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Exhibit 102



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Immunity, Inflammation, and Cancer

Sergei I. Grivennikov¹, Florian R. Greten², and Michael Karin¹

¹Laboratory of Gene Regulation and Signal Transduction, Departments of Pharmacology and Pathology, School of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA, 92093, USA.

²2nd Department of Medicine, Klinikum rechts der Isar, Technical University Munich, Munich, Germany.

Summary

Inflammatory responses play decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis. Inflammation also affects immune surveillance and responses to therapy. Immune cells that infiltrate tumors engage in an extensive and dynamic crosstalk with cancer cells and some of the molecular events that mediate this dialog have been revealed. This review outlines the principal mechanisms that govern the effects of inflammation and immunity on tumor development and discusses attractive new targets for cancer therapy and prevention.

Keywords

Cancer; inflammation; immunity; cytokines; NF- κ B; STAT3

Introduction

The presence of leukocytes within tumors, observed in the 19th century by Rudolf Virchow, provided the first indication of a possible link between inflammation and cancer. Yet, it is only during the last decade that clear evidence has been obtained that inflammation plays a critical role in tumorigenesis, and some of the underlying molecular mechanisms have been elucidated (Karin, 2006). A role for inflammation in tumorigenesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumors, including some in which a direct causal relationship with inflammation is not yet proven (Mantovani et al., 2008). Only a minority of all cancers are caused by germline mutations, whereas the vast majority (90%) are linked to somatic mutations and environmental factors. Many environmental causes of cancer and risk factors are associated with some form of chronic inflammation. Up to 20% of cancers are linked to chronic infections, 30% can be attributed to tobacco smoking and inhaled pollutants (such as silica and asbestos), and 35% to dietary factors (20% of cancer burden is linked to obesity) (Aggarwal et al., 2009).

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Corresponding author: Michael Karin karinoffice@ucsd.edu.

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Although it is now well-established that the induction of inflammation by bacterial and viral infections increases cancer risk (de Martel and Franceschi, 2009), recent work has shown that in addition to being a tumor initiator by virtue of its high carcinogen content, tobacco smoke is also a tumor promoter due to its ability to trigger chronic inflammation (Takahashi et al., 2010). Likewise, obesity, whose prevalence is growing at an alarming rate, promotes tumorigenesis in the liver (Park et al., 2010) and pancreas (Khasawneh et al., 2009). Most solid malignancies appear in older individuals and even old age (Ershler and Keller, 2000) and cell senescence (Rodier et al., 2009) are postulated to be tumor promoters that act through inflammatory mechanisms. Along with its pro-tumorigenic effects, inflammation also influences the host immune response to tumors and can be used in cancer immunotherapy (Dougan and Dranoff, 2009) and to augment the response to chemotherapy (Zitvogel et al., 2008). Yet, in some cases, inflammation can diminish the beneficial effects of therapy (Ammirante et al., 2010). This review is mainly focused on the pro-tumorigenic effects of inflammation but also touches on the relationship between inflammation and anti-tumor immunity,

Types of inflammation and general mechanisms

Several types of inflammation—differing by cause, mechanism, outcome, and intensity—can promote cancer development and progression (Figure 1). Persistent *Helicobacter pylori* infection is associated with gastric cancer and MALT (mucosa-associated lymphoid tissue) lymphoma. Infections with hepatitis B (HBV) or C (HCV) viruses increase the risk of hepatocellular carcinoma (HCC) and infections with *Schistosoma* or *Bacteroides* species are linked to bladder and colon cancer, respectively (Karin, 2006; Wu et al., 2009a). The inflammatory response triggered by infection precedes tumor development and is a part of normal host defense, whose goal is pathogen elimination. However, tumorigenic pathogens subvert host immunity and establish persistent infections associated with low grade but chronic inflammation. By contrast, acute inflammation induced by certain microbial preparations was used by Coley with some success to treat cancer in the 1890s and one such preparation is currently used in the treatment of bladder cancer (Rakoff-Nahoum and Medzhitov, 2009). What makes bladder carcinoma uniquely sensitive to acute inflammation, even though it is promoted by chronic inflammation, is currently unknown. This is an important problem whose solution should reveal how to successfully deploy inflammation in cancer therapy. Another type of chronic inflammation that precedes tumor development is caused by immune deregulation and autoimmunity. An example is inflammatory bowel disease, which greatly increases the risk of colorectal cancer (Waldner and Neurath, 2009).

However, not all chronic inflammatory diseases increase cancer risk and some of them, such as psoriasis, may even reduce it (Nickoloff et al., 2005). It is not clear what makes IBD or chronic hepatitis tumor promoting, in comparison with conditions such as rheumatoid arthritis or psoriasis, which do not significantly promote tumorigenesis. One possibility could be related to the exposure of the gastrointestinal tract and liver to dietary and environmental carcinogens, which never make their way into joints or the skin. Chronic inflammation can also be induced by environmental exposure. Particulate material from tobacco smoke and other irritants can precipitate chronic obstructive pulmonary disease, a condition associated with higher lung cancer risk (Punturieri et al., 2009). Inflammatory mechanisms account for the tumor promoting effect of exposure to tobacco smoke on lung cancer in mice (Takahashi et al., 2010). Inhaled asbestos or silica particles also give rise to lung cancer but have no obvious mutagenic activity. Such particles, however, can trigger inflammation through effects on pro-interleukin-1 β (IL-1 β) processing by the inflammasome (Dostert et al., 2008) and this may mediate their tumorigenic activity. Even obesity, which increases cancer risk by 1.6-fold (Calle, 2007), can lead to chronic inflammation (Tuncman et al., 2006) that promotes development of hepatocellular carcinoma (Park et al., 2010). Accumulation of damaged DNA and cell

senescence can also give rise to tumor promoting chronic inflammation (Rodier et al., 2009; Zheng et al., 2007).

A completely different type of inflammation is the one that follows tumor development. Most, if not all, solid malignancies trigger an intrinsic inflammatory response that builds up a pro-tumorigenic microenvironment (Mantovani et al., 2008). In addition to cell-autonomous proliferation, certain oncogenes, such as *RAS* and *MYC* family members, induce a transcriptional program that leads to remodeling of the tumor microenvironment through recruitment of leukocytes and lymphocytes, expression of tumor-promoting chemokines and cytokines, and induction of an angiogenic switch (Soucek et al., 2007; Sparmann and Bar-Sagi, 2004). All solid malignancies, at some point outpace their blood supply and become oxygen and nutrient deprived. This results in necrotic cell death at the tumor's core and the release of pro-inflammatory mediators, such as IL-1 and HMGB1 (Vakkila and Lotze, 2004). The ensuing inflammatory response promotes neo-angiogenesis and provides surviving cancer cells with additional growth factors, produced by newly recruited inflammatory and immune cells (Karin, 2006).

Other tumors, for instance lung cancer, can promote inflammation through active secretion of molecules, such as the extracellular matrix component versican that activates macrophages through Toll-like receptor (TLR) 2 (Kim et al., 2009). Based on the continuous cell renewal and proliferation induced by tumor-associated inflammation, tumors have been referred to as "wounds, which never heal" (Dvorak, 1986). This type of inflammation is largely a subverted wound healing and tissue regenerative response. Even dominant oncogenes such as v-Src or K-Ras are unable to induce cancer in adult animals unless accompanied by injury and subsequent tissue regeneration (Guerra et al., 2007; Sieweke et al., 1990).

Lastly, a strong tumor-associated inflammatory response can be initiated by cancer therapy. Radiation and chemotherapy cause massive necrotic death of cancer cells and surrounding tissues, which in turn trigger an inflammatory reaction analogous to a wound-healing response (Zong and Thompson, 2006). The net outcome of therapy-induced inflammation is controversial, as on one hand it can have tumor-promoting functions just like the necrosis that accompanies rapid tumor growth (Ammirante et al., 2010; Vakkila and Lotze, 2004), but on the other hand it can enhance the cross-presentation of tumor antigens and subsequent induction of an anti-tumor immune response (Zitvogel et al., 2008). The latter and its importance will be discussed below.

Immune cells in tumorigenesis

As a result of these different forms of inflammation, the tumor microenvironment contains innate immune cells (including macrophages, neutrophils, mast cells, myeloid derived suppressor cells, dendritic cells, and natural killer cells) and adaptive immune cells (T and B lymphocytes) in addition to the cancer cells and their surrounding stroma (which consists of fibroblasts, endothelial cells, pericytes, and mesenchymal cells) (de Visser et al., 2006) (Table 1). These diverse cells communicate with each other by means of direct contact or cytokine and chemokine production and act in autocrine and paracrine manners to control and shape tumor growth. It is the expression of various immune mediators and modulators as well as the abundance and activation state of different cell types in the tumor microenvironment that dictate in which direction the balance is tipped and whether inflammation-promotes tumor growth or anti-tumor immunity will ensue (Lin and Karin, 2007; Smyth et al., 2006). In established tumors this balance is profoundly tilted towards pro-tumor inflammation, as without therapeutic intervention advanced tumors rarely regress. Yet, it is difficult to unequivocally assess the overall impact of immunity and inflammation on early tumorigenic events, because direct in vivo models for evaluating the effects of these phenomena on initial

tumor growth are missing. In addition, our current knowledge is based on measurement of tumor load at a point where malignant cells may have already escaped early surveillance mechanisms. However, it is safe to assume that tumor promoting inflammation and anti-tumor immunity co-exist at different points along the path of tumor progression (Figure 2) and that environmental and microenvironmental conditions dictate the balance between the two (Bui and Schreiber, 2007; Swann et al., 2008).

The most frequently found immune cells within the tumor microenvironment are tumor-associated macrophages (TAMs) and T cells. TAMs mostly promote tumor growth and may be obligatory for angiogenesis, invasion, and metastasis (Condeelis and Pollard, 2006), and high TAM content generally correlates with poor prognosis (Murdoch et al., 2008). Mature T cells are divided into two major groups based on the T cell receptors (TCR) they express: $\gamma\delta$ and $\alpha\beta$. $\alpha\beta$ T cells are further classified according to their effector functions as CD8⁺ cytotoxic T cells (CTLs) and CD4⁺ helper T (Th) cells, which include Th1, Th2, Th17 and T regulatory (Treg) cells, as well as natural killer T (NKT) cells. Importantly, T cells can exert both tumor suppressive and promoting effects, as determined by their effector functions (DeNardo et al., 2009; Langowski et al., 2007; Smyth et al., 2006). Increased T cell numbers, specifically activated CTLs and Th cells, correlate with better survival in some cancers, including invasive colon cancer, melanoma, multiple myeloma, and pancreatic cancer (Galon et al., 2006; Laghi et al., 2009; Swann and Smyth, 2007). Correspondingly, T cell deficiency or disruption of specific cytotoxic mechanisms can render experimental animals more susceptible to spontaneous or chemical carcinogenesis (Shankaran et al., 2001; Swann and Smyth, 2007). However, there is also evidence that many of the T cell subsets found in solid tumors are involved in tumor promotion, progression, or metastasis, including CD8⁺ T cells (Roberts et al., 2007), IFN γ -producing Th1 cells (Hanada et al., 2006), Th2 cells (Aspord et al., 2007; DeNardo et al., 2009) and Th17 cells (Langowski et al., 2006; Wang et al., 2009). The only cells that lack a pro-tumorigenic role, so far, are NK cells. Similar to TAMs, the tumor-promoting functions of T lymphocytes are mediated by cytokines, whereas both cytokines and cytotoxic mechanisms mediate the anti-tumorigenic functions of T lymphocytes (Lin and Karin, 2007; Swann and Smyth, 2007).

Interestingly, Treg cells, which are presumed to act mostly in a pro-tumorigenic fashion through suppression of anti-tumor immune responses (Gallimore and Simon, 2008), may also exert an anti-tumorigenic function under certain circumstances by virtue of their ability to suppress tumor-promoting inflammation (Erdman et al., 2005). In breast cancer, the presence of tumor infiltrating lymphocytes with high CD4⁺/CD8⁺ and Th2/Th1 ratio is indicative of poor prognosis (Kohrt et al., 2005). Th2 CD4⁺ T cells stimulate mammary cancer progression and metastasis by educating TAMs to produce pro-angiogenic and pro-metastatic factors (DeNardo et al., 2009). In colitis associated cancer (CAC), infiltrating T cells also appear to play a tumor promoting function (Waldner and Neurath, 2009). What makes the same T cell subset anti-tumorigenic in one cancer and pro-tumorigenic in another remains largely unknown and may hold the key to the development of successful immunotherapy.

The cytokine and chemokine expression profile of the tumor microenvironment may be more relevant than its specific immune cell content. Different cytokines can either promote or inhibit tumor development and progression, regardless of their source (Lin and Karin, 2007). Through activation of various downstream effectors, such as NF- κ B, AP-1, STAT and SMAD transcription factors, as well as caspases, cytokines control the immune and inflammatory milieu to either favor anti-tumor immunity (IL-12, TRAIL, IFN γ) or enhance tumor progression (IL-6, IL-17, IL-23) and also have direct effects on cancer cell growth and survival (TRAIL, FasL, TNF- α , EGFR ligands, TGF- β , IL-6).

TAMs are one of the most important players in the inflammation and cancer arena and an important source of cytokines (Mantovani et al., 2008). In analogy to Th1 and Th2 T cells, macrophages can be classified into M1 and M2 types (Sica et al., 2008). M1 macrophages, activated by IFN γ and microbial products, express high levels of pro-inflammatory cytokines (TNF- α , IL-1, IL-6, IL-12 or IL-23), major histocompatibility complex (MHC) molecules and inducible nitric oxide synthase and are capable of killing pathogens and priming anti-tumor immune responses. By contrast, M2 or “alternatively” activated macrophages, which are induced in vitro by IL-4, IL-10 and IL-13, downregulate MHC class II and IL-12 expression and show increased expression of the anti-inflammatory cytokine IL-10, scavenger receptor A, and arginase. Most TAMs are considered to have an M2 phenotype while promoting tumor angiogenesis and tissue remodeling (Sica et al., 2008). However, most confirmed tumor-promoting cytokines are “M1 cytokines”, whereas IL-10, an M2 cytokine, may be tumor suppressive as shown in colorectal cancer (Berg et al., 1996; Lin and Karin, 2007). Furthermore, unlike Th1 and Th2 cells, M1 and M2 macrophages are plastic and their phenotype is defined by their gene expression profile rather than by deterministic differentiation pathways and lineage choices.

Other immune cells also affect tumorigenesis (Table 1). Neutrophils can play both tumor-promoting and tumoricidal functions, depending on their differentiation status and the presence of TGF- β (Fridlender et al., 2009). B lymphocytes and mast cells are also important contributors to immune-mediated tumor growth (Ammirante et al., 2010; de Visser et al., 2006; Soucek et al., 2007) and conventional macrophages and dendritic cells are important for antigen presentation and T cell activation during anti-tumor immunity as well as for cytokine production and immunosuppression in established tumors (Table 1).

Inflammation and tumor initiation

Tumor initiation is a process in which normal cells acquire the first mutational hit that sends them on the tumorigenic track by providing growth and survival advantages over their neighbors. In most cases, however, a single mutation is insufficient and many cancers require at least 4-5 mutations (Fearon and Vogelstein, 1990; Hanahan and Weinberg, 2000). It is also imperative that each mutation will be transmitted to the cell's progeny, and in cancers that arise within rapidly renewed epithelia (intestinal and skin cancers), oncogenic mutations must occur in either long lived stem cells or transient amplifying cells rather than within differentiated cells, which are rapidly eliminated before the next mutation can strike. Alternatively, oncogenic mutations can occur within differentiated epithelial cells, such as hepatocytes, which are capable of proliferation and are sufficiently long lived to allow subsequent mutational hits.

It has been suggested that an inflammatory microenvironment can increase mutation rates, in addition to enhancing the proliferation of mutated cells. Activated inflammatory cells serve as sources of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that are capable of inducing DNA damage and genomic instability (Figure 3A). However, it is not clear whether ROS and RNI produced and released by neutrophils or macrophages (mainly during acute inflammation) are sufficiently long lived to diffuse through the extracellular matrix, enter epithelial cells, cross their cytoplasm, enter the nucleus and react with DNA packaged into chromatin. Alternatively, inflammatory cells may use cytokines such as TNF- α to stimulate ROS accumulation in neighboring epithelial cells (Figure 3A). It has therefore been debated whether immune-mediated mechanisms as opposed to dietary and environmental mutagens are the critical driving forces behind tumor initiation (Hussain et al., 2003). Nonetheless, p53 mutations, presumably caused by oxidative damage, were found in both cancer cells and in inflamed, but non-dysplastic, epithelium in CAC, suggesting that chronic inflammation causes genomic changes (Kraus and Arber, 2009). Chronic inflammation triggered by the colonic irritant dextran sodium sulfate (DSS) may induce DNA damage that gives rise to colonic

adenomas (Meira et al., 2008). However, on its own DSS is a poor carcinogen (Okayasu et al., 1996).

Inflammation-induced mutagenesis may also result in inactivation or repression of mismatch repair response genes and ROS can also cause direct oxidative inactivation of mismatch repair enzymes (Colotta et al., 2009; Hussain et al., 2003). Once the mismatch repair system has been dismantled, inflammation-induced mutagenesis is enhanced and several important tumor suppressors, such as Tgfr2 and Bax, which harbor microsatellite sequences, may be inactivated (Colotta et al., 2009).

Another mechanism linking inflammation to oncogenic mutations is upregulation of AID (activation-induced cytidine deaminase), an enzyme that promotes immunoglobulin gene class switching by catalyzing deamination of cytosines in DNA (Okazaki et al., 2007). In addition to B cells, where it was discovered, AID is overexpressed in many cancers of diverse origins and its expression is induced by inflammatory cytokines in an NF- κ B-dependent manner or by TGF β (Okazaki et al., 2007). AID induces genomic instability and increases mutation probability during error-prone joining of double-stranded DNA breaks, a process found to introduce mutations into critical cancer genes, including Tp53, c-Myc, and Bcl-6 (Colotta et al., 2009). AID contributes to formation of lymphomas, and gastric and liver cancers (Okazaki et al., 2007; Takai et al., 2009). Other mechanisms of inflammation-induced mutagenesis have also been suggested, including effects of inflammation on non-homologous recombination and NF- κ B-mediated inactivation of p53-dependent genome surveillance (Colotta et al., 2009).

In *Gia2* knockout mice, which develop spontaneous colonic inflammation and cancer, enterocytes selectively lose expression of components involved in mismatch repair, namely MLH1 and PMS2, due to histone deacetylase- and DEC-1-mediated epigenetic repression of the *Mlh1* promoter (Edwards et al., 2009). Other findings implicate epigenetic mechanisms, including microRNA-based silencing and DNA methylation, in inactivation of tumor suppressors, such as INK4a and APC, and other changes that accompany tumor initiation (Cooper and Foster, 2009). Recently, inflammation has been connected to epigenetic reprogramming by the JmjC-domain protein Jmjd3, which is encoded by an NF- κ B target gene (De Santa et al., 2007). In inflammation-associated intestinal cancer in *Gpx1/2* knockout mice, inflammation induces DNA methyltransferase (DNMT)-dependent DNA methylation and silencing of a large cohort of Polycomb group target genes, some of which are also silenced by methylation in human colon cancer (Hahn et al., 2008). However, it remains to be shown that any of these inflammation-induced epigenetic mechanisms actually makes a critical contribution to tumor initiation, either in a suitable mouse model or through prospective analysis of human specimens.

Another mechanism through which inflammation may enhance tumor initiation is the production of growth factors and cytokines that can confer a stem-cell like phenotype upon tumor progenitors or stimulate stem cell expansion, thereby enlarging the cell pool that is targeted by environmental mutagens. Indeed, STAT3 is linked to both stem cell reprogramming and stem cell renewal (Chen et al., 2008), whereas NF- κ B can enhance Wnt/ β -catenin signaling in colonic crypts (Umar et al., 2009). The pro-inflammatory cytokine TNF- α promotes nuclear entry of β -catenin during inflammation-associated gastric cancer in the absence of any mutations in Wnt/ β -catenin pathway components (Oguma et al., 2008).

The connection between inflammation and tumor initiation is not a one-way street and there is also evidence that DNA damage can lead to inflammation and thereby promote tumorigenesis. One of the best examples is provided by the model of hepatocellular carcinoma induced by the carcinogen diethylnitrosamine (DEN) in which DNA damage contributes to necrotic cell death, resulting in an inflammatory reaction that promotes tumor development

(Maeda et al., 2005; Sakurai et al., 2008). A number of oncoproteins (Ras, Myc, RET) can activate signaling pathways that drive production of pro-inflammatory cytokines and chemokines (IL-6, IL-8, IL-1 β , CCL2, CCL20) (Mantovani et al., 2008). Genotoxic stress can also induce expression of NKG2D family members, which serve as ligands for NK and $\gamma\delta$ T cell receptors (Strid et al., 2008) resulting in either elimination of stressed cells or a local inflammatory response. In the same vein, mosaic deletion of the DNA repair gene ATR and Tp53 in the skin results in recruitment of CD11b⁺Gr1⁺ myeloid cells, as a part of a prototypical immune response to “altered self” (Ruzankina et al., 2009). Defective DNA repair caused by a deficiency of the Fen1 exonuclease also results in a tumor promoting inflammatory response that is driven by damaged DNA, most likely through activation of a pattern recognition receptor (Zheng et al., 2007).

Inflammation and tumor promotion

Tumor promotion is the process of tumor growth from a single initiated cell into a fully developed primary tumor. Initial tumor growth depends on increased cell proliferation and reduced cell death, both of which are stimulated by inflammation-driven mechanisms. In fact, many of the enhancing effects of inflammation on cancer are exerted at the level of tumor promotion and most known tumor promoters, for instance phorbol esters, are potent inducers of inflammation (Karin, 2006). Inflammation-induced tumor promotion may occur early or late in tumor development and can lead to activation of pre-malignant lesions that were dormant for many years. The mechanisms through which inflammation affects tumor promotion are numerous and in addition to increased proliferation and enhanced survival, can also involve the so-called angiogenic switch, which allows a small dormant tumor to receive the blood supply necessary for the next growth phase (Lewis and Pollard, 2006). Mechanisms of inflammation-driven tumor promotion are discussed below.

Tumor promoting cytokine signaling

Production of tumor promoting cytokines by immune/inflammatory cells that activate transcription factors, such as NF- κ B, STAT3 and AP-1, in pre-malignant cells to induce genes that stimulate cell proliferation and survival, is a major tumor promoting mechanism (Figure 3B). Initial evidence for inflammation-mediated tumor promotion came from mouse models of skin, colon, and liver cancer. Although counterintuitive at the time, TNF- α was found to be required for two-stage skin carcinogenesis (Moore et al., 1999). TNF- α activates both AP-1 and NF- κ B transcription factors, but in the skin its tumor promoting effects are mediated by AP-1 (Eferl and Wagner, 2003), which was identified as a transcription factor whose activity is stimulated by the classic tumor promoter tetradecanoyl phorbol acetate (TPA) (Angel et al., 1987). By contrast, NF- κ B inhibits the development of skin cancer (Zhang et al., 2004). Thus, although a given cytokine may activate several transcription factors, its tumor promoting activity may be mediated by only one of them and antagonized by another. As discussed below, a similar situation may apply to liver cancer. Amongst the different transcription factors that are part of this mechanism, NF- κ B and STAT3 are activated in the majority of cancers and act as non-classical oncogenes, whose activation in malignant cells is rarely the result of direct mutations, and instead depends on signals produced by neighboring cells or more rarely on mutational activation of upstream signaling components. NF- κ B and STAT3 activate genes that control cell survival, proliferation, and growth, as well as angiogenesis, invasiveness, motility, chemokine, and cytokine production (Grivennikov and Karin, 2009; Yu et al., 2009).

Oncogenic transcription factors can also be activated through pattern recognition receptors by components of bacteria and viruses (Rakoff-Nahoum and Medzhitov, 2009). However, the overall contribution of pattern recognition receptors on epithelial cells versus those expressed

by immune/inflammatory cells to tumor promotion is far from being clear and will require the analysis of cell type specific knockout mice. Even the specific agonists that activate these receptors in cancer are not defined. Nonetheless, the role of the cytokines that are produced in response to damage-associated (DAMP) or pathogen-associated (PAMP) molecular patterns in tumor development is more firmly established. For example, AP-1 activation in skin cancer is largely dependent on TNF-TNFR1 signaling (Balkwill, 2009), whereas STAT3 activation in cancer cells is largely dependent on a plethora of growth factors and cytokines including IL-6, IL-11, IL-22, HGF, and EGF, and oncogenic tyrosine kinases, such as c-Met and Src (Bollrath et al., 2009; Grivennikov et al., 2009; Naugler et al., 2007; Yu et al., 2009).

The first critical genetic evidence for inflammatory cells as a source of tumor promoting cytokines was obtained in a mouse model of CAC, where inactivation of NF- κ B in myeloid cells reduced tumor growth and blocked production of IL-6 and other cytokines in response to colitis (Greten et al., 2004). Subsequent work demonstrated that the effect of immune cells (macrophages, T cells) on CAC growth is mediated through IL-6, IL-11, TNF- α and IL-1 β (Becker et al., 2004; Bollrath et al., 2009; Grivennikov et al., 2009; Popivanova et al., 2008), as well as other cytokines, such as IL-23. IL-11 plays a similar role in gastric cancer (Ernst et al., 2008), in which IL-1 β is also a tumor promoter (Tu et al., 2008). TNF- α also promotes HCC in mice lacking the P-glycoprotein Mdr2, which develop cholestatic inflammation followed by hepatocellular carcinoma (HCC) (Pikarsky et al., 2004). HCC can also be promoted by another member of the TNF family, lymphotoxin β (Haybaeck et al., 2009). TNF- α along with IL-6 contributes to obesity-mediated tumor promotion in HCC (Park et al., 2010). The latter effect correlates with the ability of TNF- α and IL-6 to promote hepatosteatosis and steatohepatitis (Park et al., 2010). One of the most critical tumor promoting cytokines in HCC is IL-6. Mice deficient in IL-6 develop much less HCC in response to the chemical pro-carcinogen DEN and the gender-biased production of IL-6 accounts for the much higher HCC load in males (Naugler et al., 2007). High levels of circulating IL-6, are associated with HCC risk factors, including hepatosteatosis, obesity, and liver cirrhosis, and are the best predictors of rapid progression from viral hepatitis to HCC in humans (Wong et al., 2009).

In CAC and HCC, the tumor promoting effect of IL-6 is mainly exerted via STAT3, whose cell type specific inactivation in hepatocytes and enterocytes inhibits the development of these malignancies in mice treated with DEN or azoxymethane (AOM) and DSS, respectively (Bollrath et al., 2009; Grivennikov et al., 2009; Park et al., 2010). Development of CAC in mice is also dependent on IKK β -mediated NF- κ B activation in enterocytes, whose major function in this model is increased survival of pre-malignant cells (Greten et al., 2004). A similar role was proposed for NF- κ B in HCC development in mice deficient in *Mdr2* and in lymphotoxin-transgenic mice both of which exhibit chronic liver inflammation (Haybaeck et al., 2009). However, in the DEN model of HCC and *Helicobacter*-driven gastric cancer, NF- κ B promotes hepatocyte and epithelial cell survival and acts as an inhibitor of tumor development (Maeda et al., 2005; Shibata et al., 2009). Most likely, the diverse effects of NF- κ B in different models are determined by the mechanism of tumor induction and the type of inflammatory response involved in tumor promotion. *Mdr2* knockout and lymphotoxin-transgenic mice exhibit a very low level of normal hepatocyte death, which is not enhanced by the absence of NF- κ B (Haybaeck et al., 2009; Pikarsky et al., 2004). In these mice, NF- κ B in hepatocytes is mainly responsible for propagating inflammation through induction of chemokines, which recruit immune/inflammatory cells into the liver. By contrast, DEN treated mice exhibit an acute inflammatory response triggered by IL-1 α release from necrotic hepatocytes (Sakurai et al., 2008). IL-1 α induces IL-6 production by Kupffer cells and this response drives the compensatory proliferation of surviving hepatocytes (a type of a wound-healing response); the greater the amount of cell death – the greater the regenerative response. By suppressing accumulation of ROS and preventing hepatocyte necrosis, NF- κ B inhibits HCC induction in DEN treated mice (Maeda et al., 2005).

Another tumor-promoting cytokine is IL-23 (Langowski et al., 2006). IL-23 is mostly expressed by TAMs in a manner dependent on STAT3 and NF- κ B (Kortylewski et al., 2009). IL-23 blockade with neutralizing antibodies or genetic inactivation of the IL-23p19 gene dramatically decrease tumor multiplicity and growth in the two-step model of skin carcinogenesis (Langowski et al., 2006). In part, the pro-tumorigenic effects of IL-23 may be mediated by IL-17 and IL-22 production by Th17 cells, but other effects of IL-23 on CTLs, Tregs, and myeloid cells should not be discounted. A close relative of IL-23 is IL-12, which shares with IL-23 the IL-12p40 subunit and is involved in Th1 differentiation, IFN γ production, and activation of anti-tumor immunity (Trinchieri et al., 2003). Secretion of IL-23 and IL-12 secretion are reciprocally regulated and the switch from IL-12 to IL-23 production may be an important tumor promoting event. STAT3 activation, PGE₂, ATP, and lactic acid increase IL-23 production by TAMs (Kortylewski et al., 2009; Shime et al., 2008). The latter two agonists link cancer cell necrosis (induced by hypoxia or therapy) and the Warburg effect (the switch from oxidative phosphorylation to glycolysis) to IL-23 production, thereby shifting anti-tumor immunity to tumor promotion.

A similar circuit can be executed by myeloid-derived suppressor cells (MDSC) that produce arginase1 and indoleamine-2,3-dioxygenase, which are enzymes that dampen anti-tumor immunity through interference with T cell activation (Gabrilovich and Nagaraj, 2009). Taken together, tumor associated inflammation drives tumor growth, angiogenesis and can perpetuate itself through an extensive network of cytokines and chemokines, which are produced by immune, stromal and malignant cells in response to diverse signals (Figure 3B).

Given that several cytokines (IL-1, TNF, IL-6, IL-23) and transcription factors (AP-1, NF- κ B, STAT3) are critical for both inflammation and tumor growth, they control hubs of pro-tumorigenic signaling that may be targeted to curtail both tumor associated inflammation and tumor growth (see below). Pharmacological interference with cytokine signaling decreases tumorigenesis as well as cancer growth (Becker et al., 2004; Grivennikov et al., 2009; Hedvat et al., 2009) and may therefore serve as a basis for preventive and therapeutic approaches. Altogether, cytokine production by immune and inflammatory cells is an important tumor promoting mechanism that provides malignant cells with a continuous supply of growth and survival signals in an initially hostile microenvironment. In most cases, tumor promoting cytokines act in a paracrine manner, yet several types of cancer cells produce their own cytokines, including IL-6, to achieve the same effect (Gao et al., 2007).

Inflammation and angiogenesis

Growth of large tumors requires an increased intratumoral blood supply. This is triggered by tumor hypoxia, which promotes angiogenesis and increases the probability of metastasis. In addition to hypoxia, tumor angiogenesis depends on recruitment of TAMs, which sense hypoxic signals and in turn produce chemokines and pro-angiogenic factors. Recruitment of TAM precursors is largely dependent on angiogenic mediators such as angiopoietin 2 and vascular endothelial growth factor (VEGF). Important pro-angiogenic genes, such as IL-8, CXCL1, CXCL8, VEGF and hypoxia inducible factor 1 alpha (HIF1 α), are directly regulated by NF- κ B, STAT3 and AP-1 in TAMs, MDSCs, and other cell types (Kujawski et al., 2008; Rius et al., 2008).

Under hypoxic conditions, HIF-1 α stimulates expression of CXCL12, which activates and recruits endothelial cells in a CXCR4-dependent manner (Sica et al., 2008). Formation of new lymphatic vessels is regulated by VEGF-C and VEGF-D, whereas VEGF-A facilitates the recruitment of monocytes, which activate lymphoangiogenesis (Murdoch et al., 2008). VEGF-A produced by myeloid cells also inhibits pericyte maturation and endothelial coverage of newly formed blood vessels, and its conditional ablation accelerates tumorigenesis (Stockmann

et al., 2008). The recruitment of Gr1⁺ myeloid cells (presumably MDSC and TAM precursors) into tumors, curtails the effects of anti-VEGF therapy, presumably bypassing the requirement for local VEGF production by cancer cells for recruitment of TAM precursors (Shojaei et al., 2007). As most growing tumors contain some areas of hypoxia, it is not clear whether hypoxia is the direct driver of tumor angiogenesis or whether hypoxic stimuli generate inflammatory signals that drive angiogenesis. Inactivation of NF- κ B or STAT3, neutralization of CCL2 or CXCL12, or TAM depletion unequivocally result in disrupted angiogenesis and decreased tumor growth, underscoring the critical role of inflammatory mediators in tumor angiogenesis (Joyce and Pollard, 2009; Kujawski et al., 2008).

Target genes that mediate tumor promotion

Most of the genes that mediate the tumor promoting functions of NF- κ B, STAT3, and AP-1 have not been fully defined and most likely the pro-tumorigenic effects of these transcription factors are exerted through multiple effectors. Some targets may be controlled by more than one transcription factor and may be more important in one cell type than in another. The expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, for instance, are promoted by both NF- κ B and STAT3 as are c-IAP1, c-IAP2, Mcl-1, c-FLIP, and survivin (Karin, 2006; Yu et al., 2007). Whereas Bcl-X_L may be the most prominent anti-apoptotic gene in enterocytes (Greten et al., 2004), c-FLIP seems to fulfill the same function in hepatocytes (Chang et al., 2006). Both NF- κ B and STAT3 interfere with p53 synthesis and attenuate p53-mediated genomic surveillance, representing another potential tumor promoting mechanism (Colotta et al., 2009).

STAT3 controls expression of cyclins D1, D2 and B, as well as the proto-oncogene c-Myc, and through them it may stimulate cell proliferation (Bollrath et al., 2009; Yu et al., 2007). Although cyclin D and c-Myc are also thought to be regulated by NF- κ B, inactivation of IKK β in enterocytes does not interfere with cell proliferation (Greten et al., 2004) and in Rastransformed keratinocytes (Zhang et al., 2004) or DEN-initiated hepatocytes (Maeda et al., 2005) NF- κ B inhibition actually enhances cyclin D expression and cell proliferation. The AP-1 protein c-Jun cooperates with STAT3 in repression of Fas expression by tumor cells, thereby attenuating their sensitivity to instructive apoptosis (Eferl and Wagner, 2003). Additional NF- κ B and STAT3 targets control cell and tissue resistance to stress and injury and include anti-microbial proteins (RegIII β , RegIII γ , Tff3), heat shock proteins, and anti-oxidants, such as superoxide dismutase 2 (SOD2) and ferritin heavy chain (FHC) (Bollrath et al., 2009; Karin, 2006).

Lastly, another category of target genes that promote tumorigenesis are chemokines and cytokines that act in autocrine or paracrine manners to ensure the continuous recruitment of inflammatory cells into the tumor microenvironment. The perpetuation of chronic inflammation is largely achieved through positive feedback loops, which include inflammatory cells producing cytokines that induce chemokine synthesis in malignant and stromal cells leading to prolonged recruitment of inflammatory cells into the tumor microenvironment (Figure 3). TAMs, MDSCs, Tregs, and Th17 cells are the most critical immune cell subsets in this respect. Recruitment of myeloid cells is governed by multiple pathways, including CCL2-CCR2, CCL1-CXCR2, S100A proteins-RAGE, and IL-1-IL-1R interactions (Bonecchi et al., 2009). Signaling through CCR6 is critical for Th17 infiltration, whereas Treg cells are attracted mostly through CCR4 and CCR7 (Bonecchi et al., 2009). In some cases, the critical chemokines are not produced by cancer cells but are induced in tumor-associated fibroblasts upon interaction with carcinoma cells (Liao et al., 2009; Orimo et al., 2005; Orimo and Weinberg, 2006).

Inflammation and lymphoid malignancies

Chronic inflammatory conditions are also associated with lymphoid malignancies. An excellent example is provided by mucosa-associated lymphoid tissue (MALT) lymphomas, which occur in the context of chronic inflammation caused by infectious agents, such as *Helicobacter pylori* (the most commonly found gastric lymphoma), *Chlamydia psittacii* (ocular adnexal MALT lymphoma) and *Borrelia burgdorferi* (cutaneous MALT lymphoma) (Ferrerri et al., 2009). Another example is Epstein-Barr virus (EBV), which is responsible for large B-cell lymphoma in immunocompromised patients, Burkitt's lymphoma, and Hodgkin's lymphoma (Ferrerri et al., 2009).

It has been proposed that repeated antigenic stimulation, autoimmunity, and inflammation are risk factors for chronic lymphocytic leukemia (CLL), the most common hematopoietic malignancy that accounts for 30% of all leukemias (Chiorazzi et al., 2005). One mechanism through which such stimuli promote CLL development is induction of B cell activating factor (BAFF), a member of the TNF family, recently shown to accelerate development of CLL-like disease in mice (Enzler et al., 2009). Cytokines (such as IL-4 and VEGF), chemokines (such as SDF-1), and interactions with bone marrow stromal cells support CLL expansion and suppress apoptosis through upregulation of Bcl-2, survivin, and MCL-1 (Granziero et al., 2001; Pedersen et al., 2002). This occurs in lymph node pseudofollicles and bone marrow clusters where leukemic cells interact with components of the inflammatory microenvironment that support their survival. Another example for the role of inflammation in lymphoid malignancies are the lymphomas that appear in GM-CSF- and IFN γ -deficient mice, which are caused by infections and regress upon treatment with antibiotics (Enzler et al., 2003).

A similar situation may occur in multiple myeloma. Through secretion of IL-6, IGF-1, VEGF, TNF- α , SDF-1 and BAFF, stromal elements promote the survival and migration of neoplastic plasma cells and also confer drug resistance (Kastritis et al., 2009). IL-6 is of particular importance, as it acts both in paracrine and autocrine manners and IL-6-deficient mice are resistant to induction of multiple myeloma (Hodge et al., 2005). Despite constitutive NF- κ B activation, multiple myeloma remains dependent on extrinsic factors, and drugs targeting IL-6 are being evaluated in combination with the proteasome inhibitor bortezomib for the treatment of this malignancy (Kastritis et al., 2009).

Inflammation and metastasis

From a clinical perspective, metastasis is the most critical aspect of tumorigenesis, because over 90% of cancer mortality is caused by metastasis. Recent studies unambiguously show that metastasis requires close collaboration between cancer cells, immune and inflammatory cells, and stromal elements. The process of metastasis can be grossly divided into four major steps. The first step is represented by epithelial-mesenchymal transition, in which cancer cells acquire fibroblastoid characteristics that increase their motility and allow them to invade epithelial linings/basal membranes and reach efferent blood vessels or lymphatics (Kalluri and Weinberg, 2009). Loss of E-cadherin expression is envisioned as a key event in the epithelial-mesenchymal transition. In the second step, cancer cells intravasate into blood vessels and lymphatics. Inflammation may promote this through production of mediators that increase vascular permeability. This is followed by the third step in which metastasis initiating cells survive and travel throughout the circulation. It has been estimated that only about 0.01% of cancer cells that enter the circulation will eventually survive and give rise to micrometastases (Joyce and Pollard, 2009). Next, integrin-mediated arrest allows the extravasation of circulating cancer cells. Finally, single metastatic progenitors interact with immune, inflammatory, and stromal cells and start to proliferate (Polyak and Weinberg, 2009). Some of these cells may already be targeted to the pre-metastatic niche in response to tumor generated

inflammatory signals prior to the arrival of metastasis-initiating cancer cells (Kaplan et al., 2005). One of these inflammatory signals is the extracellular matrix component versican, which leads to macrophage activation and production of the metastasis promoting cytokine TNF- α (Kim et al., 2009). However, it has been difficult to determine whether versican production by metastatic cancer cells conditions the future metastatic site prior to their arrival.

TGF β is an anti-inflammatory cytokine produced by cancer cells, myeloid cells, and T lymphocytes. TGF β signaling is an important regulator of the epithelial-mesenchymal transition and metastasis, and elevated TGF β is often associated with poor prognosis (Yang and Weinberg, 2008). TGF β activates SMAD transcription factors and MAPKs, which control expression of other regulators of the epithelial-mesenchymal transition, such as Slug (Yang and Weinberg, 2008). TGF β however, also suppresses epithelial cell proliferation and early tumor growth, causing some tumors to acquire inactivating mutations in TGF β signaling components (Yang and Weinberg, 2008). Despite the defects in TGF β signaling, such tumors can still metastasize. These opposing effects of TGF β at different stages of tumor development await mechanistic explanation. Disruption of TGF β signaling in cancer cells also results in upregulation of the SDF1 (CXCL12)-CXCR4 and CXCL5-CXCR2 chemokine:chemokine receptor pairs and induces rapid recruitment of MDSCs that promote metastasis and dampen anti-tumor immune responses (Yang et al., 2008). Inactivation of TGF β signaling was proposed to result in elevated local TGF β concentrations that inhibit anti-tumor T cell responses and induce differentiation of tumor-promoting Th17 cells (Langowski et al., 2007).

Another critical regulator of the epithelial-mesenchymal transition is Snail, a repressor of E-cadherin transcription in epithelial cells. Recent findings suggest that Snail is stabilized in response to TNF- α signaling, a process that is critical for cancer cell migration and metastasis (Wu et al., 2009b). Other mechanisms through which pro-inflammatory cytokines can affect the epithelial-mesenchymal transition is via STAT3-mediated induction of Twist transcription and NF- κ B-mediated induction of both Twist and Kiss (Yu et al., 2009). However, these mechanisms remain to be confirmed in vivo, and a recent report suggests that STAT3 is a negative regulator of adenoma-carcinoma transition in colon cancer (Musteanu et al., 2009).

Cancer cell invasion requires extensive proteolysis of the extracellular matrix at the invasive front. Inflammatory cells are important sources of proteases that degrade the extracellular matrix. In a model of invasive colon cancer, CCR1⁺ myeloid cells, whose recruitment is driven by the chemokine CCL9 produced by cancer cells, promote invasiveness through secretion of the matrix metalloproteinases MMP2 and MMP9 (Kitamura et al., 2007). IL-1, TNF- α and IL-6 promote MMP expression, invasiveness, and metastasis via NF- κ B and STAT3 (Yu et al., 2007).

A different metastatic mechanism dependent on IKK α operates in prostate and breast cancers. As these cancers progress, their malignant cells progressively accumulate activated IKK α in their nuclei (Luo et al., 2007). In prostate cancer, accumulation of activated nuclear IKK α correlates with reduced expression of maspin, an inhibitor of metastasis (Luo et al., 2007). IKK α activation in metastatic prostate and mammary cancer cells is mediated by members of the TNF family, namely lymphotoxin and RANKL and its repressive effects on maspin transcription are NF- κ B independent (Luo et al., 2007). How these lymphocytes are recruited into progressing breast and prostate tumors is still unknown. Recruitment of such cells may be a consequence of tumor necrosis, but as mentioned above certain carcinomas actively secrete factors that upregulate fibronectin and cause migration of VEGF receptor 1 (VEGFR1)-positive hematopoietic progenitors to the pre-metastatic niche (Kaplan et al., 2005). However, the pre-metastatic niche concept is somewhat mysterious as it is not clear how primary tumor cells direct inflammatory cells to such sites.

Alternatively, a small number of metastatic cells can interact with and activate different myeloid cell types through secreted factors such as versican (Kim et al., 2009). Breast cancer cells use CSF1 and CXCL12 to induce the recruitment of TAMs, which in turn produce EGF receptor (EGFR) ligands (Joyce and Pollard, 2009). These cytokines may also mediate a physical interaction between TAMs and carcinoma cells (Condeelis and Pollard, 2006). TAMs can be also “programmed” by tumor infiltrating T cells, particularly Th17 cells (Wang et al., 2009) and Th2 cells (DeNardo et al., 2009). IL-13 and IL-4 produced by tumor infiltrating CD4⁺ T cells stimulate the M1 to M2 transition of TAMs and thereby support pulmonary metastasis of mammary cancer cells (DeNardo et al., 2009). Depletion of TAMs (Joyce and Pollard, 2009) or CD4⁺ T cells (DeNardo et al., 2009) dramatically reduces metastasis of mouse mammary cancer.

Once metastatic cells enter the circulation, they need to survive in suspension and resist detachment-induced cell death or anoikis. The survival of circulating cancer cells is affected by inflammatory mediators released by immune cells in response to cancer-derived or pathogen-derived stimuli (Kim et al., 2009; Luo et al., 2004). Some of these effects depend on activation of NF- κ B in either inflammatory cells or in cancer cells. A variety of cytokines present in the tumor microenvironment, including TNF- α , IL-6, and epiregulin, can promote the survival of circulating metastatic seeds (Nguyen et al., 2009). In addition to NF- κ B and STAT3 activation, some of these cytokines can physically link cancer cells to TAMs, allowing them to travel together throughout the circulation (Condeelis and Pollard, 2006). On the other hand, single metastatic cells, which are no longer present within an immunosuppressive environment, may be targeted again by immunosurveillance. Indeed, in some cases, infiltration of tumors by activated T cells decreases the rate of metastasis (Galon et al., 2006; Pages et al., 2005). The interaction of circulating cancer cells with platelets or macrophages may protect them from NK cell-mediated killing, thereby overcoming immunosurveillance (Palumbo et al., 2007).

Intravasation is regulated by prostaglandins (which are produced in a COX2-dependent manner and act on the epithelium), by cytokines (such as epiregulin, which increases cancer cell survival), and by MMPs (which clear the way for the latter to migrate into capillaries (Nguyen et al., 2009)). The migration of metastasis initiating cells is not random and is directed by chemokine gradients sensed via CXCR4, CCR4, CCR7, CCR9 and CCR10 (Bonecchi et al., 2009).

The journey of the circulating metastatic seed ends upon integrin-dependent arrest on the endothelium, followed by extravasation. Molecules like ANGPTL4, which is regulated by TGF β , facilitate extravasation into lungs by mediating contact between malignant and endothelial cells (Nguyen et al., 2009). Systemic inflammation enhances attachment of circulating cancer cells to hepatic sinusoids and this process is governed by neutrophil-dependent upregulation of adhesion molecules (McDonald et al., 2009). Several proinflammatory cytokines that are elevated in the circulation of cancer patients upregulate expression of adhesion molecules on the endothelium or in target organs and thereby increase the probability of metastatic cell attachment (Mantovani et al., 2008).

Immunity and tumorigenesis

As discussed above, in tumors that arise in the context of underlying inflammation or in advanced tumors containing inflammatory infiltrates, the net effect of the immune system (both innate and adaptive) is stimulation of tumor growth and progression. However, cancer cells represent an “altered self” and express “non-self” antigens in the context of stress and danger signals that can promote antigen presentation. Thus, even growing tumors may be subject to immunosurveillance and killing by activated T and NK cells (Dunn et al., 2004). It is likely

that immunosurveillance and tumor-promoting inflammation can coexist even in the same tumor (Bui and Schreiber, 2007) (Figure 4A).

According to the immunosurveillance hypothesis, NK cells and CTLs engage in tumor killing (via perforin, granzyme B, TRAIL or FasL dependent mechanisms), whereas Th1 (by virtue of IFN γ production) and in some instances Th17 cells (via production of IL-17A) provide important help that boosts cytotoxic immunity (Dunn et al., 2006; Dunn et al., 2004; Martin-Orozco et al., 2009). On the other hand, Tregs suppress anti-tumor immune responses and are therefore pro-tumorigenic (Dunn et al., 2004). NKT cells can also be involved in surveillance of hematopoietic and chemically-induced tumors (Crowe et al., 2005; Smyth et al., 2000; Swann et al., 2009). Other critical components of this system are dendritic cells and macrophages, which present antigens and respond to danger and stress signals, as well as immunoregulatory and cytotoxic cytokines, such as type I IFN, IFN γ , FasL, TRAIL, GM-CSF and IL-12 (Palucka et al., 2007; Smyth et al., 2006; Swann and Smyth, 2007).

The first experimental demonstration of tumor immunosurveillance came from analysis of Rag2-deficient mice, which lack mature lymphocytes. These mice show enhanced development of a variety of spontaneous cancers by 14-16 months of age (Shankaran et al., 2001). However, even in immunocompromised mice, tumor development occurs in their post-reproductive period, suggesting that the mammalian immune system is not subjected to substantial evolutionary pressure to improve tumor recognition and elimination. Yet, in virally or bacterially-promoted cancers, the immune system provides considerable protection through its ability to recognize and eliminate microbes (Smyth et al., 2006). Inactivation of various components of the immunosurveillance system, such as perforin, granzyme, and interferon signaling, renders mice susceptible to tumorigenesis (Bui and Schreiber, 2007; Dunn et al., 2004). Mice lacking cytotoxic cytokines, such as membrane-bound forms of FasL or TRAIL also show enhanced development of sarcomas and other tumors (O'Reilly et al., 2009; Smyth et al., 2003).

More evidence for tumor immunosurveillance and immunoediting comes from the presence of tumor infiltrating lymphocytes (both T and B lymphocytes) that recognize tumor antigens and the favorable prognosis for some patients whose tumors display increased infiltration with activated T cells (Dunn et al., 2004). Such infiltration is even more noticeable in tumors that develop microsatellite instability or have a "mutator" phenotype and therefore express tumor antigens that exhibit greater differences from normal counterparts (Buckowitz et al., 2005; Guidoboni et al., 2001). Additional but indirect evidence for anti-tumor immunity includes various cases of spontaneous tumor regression accompanied by increased infiltration of activated cytotoxic cells and presence of antibodies and T cells that recognize tumor antigens (Swann and Smyth, 2007). The latter suggests that B and T lymphocytes have been activated by tumor-specific antigens but does not necessarily mean that these cells are responsible for tumor regression. Additional evidence is provided by the increased risk of lymphomas (of viral and non-viral etiology) and some solid tumors in immunosuppressed patients (Swann and Smyth, 2007).

Nonetheless, in the vast majority of established tumors the presence of tumor infiltrating lymphocytes is insufficient for curtailing tumor growth. Such considerations have given rise to a revised version of the immunosurveillance theory called immunoediting (Dunn et al., 2004; Smyth et al., 2006). According to this concept, cancer cells constantly edit and modulate the host anti-tumor immune response and the host immune response shapes tumor immunogenicity and clonal selection. During this process the balance between anti-tumor and tumor-promoting immunity can be tilted in favor of tumor growth. Before a tumor undergoes immune escape, it may be maintained at an "equilibrium" between tumor growth and immune destruction, and this may account for decades of tumor dormancy (Koebel et al., 2007). To tilt

the balance in its favor, it is proposed that the cancer cell edits its repertoire of tumor antigens towards lower immunogenicity and also re-shapes the tumor microenvironment to become immunosuppressive. Consistent with this hypothesis, cancers that have evolved in alymphocytic mice are more immunogenic than cancers grown in immunocompetent mice (Shankaran et al., 2001).

Therapy induced inflammation – friend or foe?

Surgery, chemotherapy, and radiation are currently the major options for cancer treatment. All three induce local or systemic inflammation triggered by tissue injury and cancer cell death. Surgery results in activation of infection or stress-sensing pathways, whereas chemo- and radiotherapy kill cancer cells mostly through necrosis, a pro-inflammatory form of cell death (Vakkila and Lotze, 2004). Inflammatory mediators released by necrotic cells include danger associated molecular patterns (DAMPs) such as ATP, nucleic acids, heat shock proteins (Hsp70), HMGB-1, S100 calcium binding proteins, and the cytokine IL-1 α . A key question is whether therapy-induced inflammation stimulates the regrowth of residual malignant cells or whether it improves the therapeutic outcome? (Figure 4B). In support of the first possibility, inhibition of autophagy in apoptosis-deficient tumors stimulates tumor growth through induction of necrosis and tumor-associated sterile inflammation (Degenhardt et al., 2006). Tumor growth may also be stimulated in response to hypoxia-induced necrosis in the tumor's core (Figure 4B). It has also been found that castration-induced death of androgen-dependent prostate cancer, despite resulting in initial tumor regression, triggers an inflammatory response that accelerates the re-growth of castration resistant cancer (Ammirante et al., 2010). Hence, inhibition of therapy-induced inflammation may improve the treatment of prostate cancer and provide the patient with several more years of tumor free survival.

However, in the case of more conventional chemotherapy, therapy-induced inflammation has been found to stimulate antigen presentation by tumor infiltrating dendritic cells and to induce production of cytokines that stimulate adaptive anti-tumor immunity (Apetoh et al., 2007a; Zhang et al., 2007) (Figure 4B). Curiously, the inflammatory trigger for this beneficial response is also the necrotic death of cancer cells, resulting in the release of HMGB-1 and ATP, which together activate TLR4 and the inflammasome to stimulate production of IL-1 β , which is critical for adaptive anti-tumor immunity (Ghiringhelli et al., 2009). Interestingly, genetic polymorphisms in the TLR4 and P2X7 (the ATP receptor) loci affect the outcome of chemotherapy (Apetoh et al., 2007a; Apetoh et al., 2007b). What makes tumor necrosis either immunostimulatory or immunosuppressive (Vakkila and Lotze, 2004) is not yet clear. Furthermore, therapy-induced anti-tumor immunity is only seen with certain drugs, including etoposide, oxaliplatin, and doxorubicine but not with others (Apetoh et al., 2007a; Ghiringhelli et al., 2009). As these drugs can also kill infiltrating immune and hematopoietic stem cells, which are necessary for a functional immune response, effective therapy-induced anti-tumor immunity requires the use of small doses of chemotherapy to avoid immunosuppression. Conversely, by causing the death of tumor promoting immune/inflammatory cells, chemo- and radiotherapy may be used to destroy the tumor-promoting inflammatory microenvironment.

Anti-inflammatory drugs in cancer therapy

The findings described above provide an improved understanding of the molecular etiology of cancer and lay the foundations for the use of anti-inflammatory drugs in cancer prevention and therapy. One advantage of targeting the inflammatory microenvironment is that the normal genome of inflammatory/immune cells, which unlike the cancer cell genome, is not subject to mutational and epigenetic changes that result in drug resistance. However, in most cases, anti-inflammatory therapy is not cytotoxic on its own and needs to be combined with more conventional therapies that kill cancer cells.

Despite such limitations, several anti-inflammatory drugs have been found to reduce tumor incidence when used as prophylactics, as well as slowing down progression and reducing mortality when used as therapeutics, particularly in the case of sporadic colon cancer (Gupta and Dubois, 2001). Such drugs include COX2 inhibitors, aspirin, and anti-inflammatory steroids, such as dexamethasone. In addition to its well-documented preventive effects in colon cancer, aspirin reduces the incidence of breast cancer (Gierach et al., 2008) and reduces prostate cancer risk, but only in individuals that carry a particular polymorphic allele at the lymphotoxin α locus, which specifies high lymphotoxin production (Liu et al., 2006). Such findings are of general importance because non-steroidal anti-inflammatory drugs (NSAID), such as aspirin, are not very specific and usually have side-effects that preclude their long-term administration except in high risk individuals. Thus, pre-screening for individuals with high cancer risk that are more likely to benefit from such preventive strategies should greatly improve the efficacy and utility of cancer prevention.

Tumor-promoting inflammation can be targeted in several different ways: 1) inhibition of signal transducers and transcription factors that mediate survival and growth of malignant cells in response to inflammatory cytokines; 2) sequestration of chemokines and cytokines that recruit and sustain inflammatory cells in the tumor microenvironment; 3) reducing (or augment) the inflammation that follows anti-cancer therapy; 4) depletion of immune and inflammatory cells that promote tumor development and progression, while sparing cell types and effector functions that support protective immune responses; 5) selective inhibition of tumor promoting cytokines without an effect on expression of anti-tumorigenic cytokines.

In a few cases, a therapy targeting inflammation may be effective as a single agent. For instance, constitutive NF- κ B or STAT3 activation in certain lymphoid tumors suggests that inhibitors of these transcription factors can be used as cytotoxic agents in such cancers. However in most cases such therapy is likely to be effective only in combination with more conventional approaches. Furthermore, as genotoxic therapies often lead to NF- κ B activation in remaining malignant cells, it makes sense to combine genotoxic drugs with NF- κ B inhibitors as a way to overcome drug resistance. However, prolonged NF- κ B inhibition can result in a severe immune deficiency and may even lead to neutrophilia and greatly enhanced acute inflammation due to enhanced IL-1 β secretion (Greten et al., 2007). Such complications as well as increased propensity for liver damage have hindered the clinical development of NF- κ B and IKK β inhibitors. Another attractive target is the STAT3 transcription factor and the signaling pathway that leads to its activation (Kortylewski et al., 2005; Yu et al., 2009). Several STAT3 and JAK2 inhibitors have been described and shown to inhibit the growth of various cancers that exhibit STAT3 activation (Hedvat et al., 2009; Lin et al., 2009). So far, none of the complications associated with NF- κ B inhibition have been reported for STAT3 or JAK2 inhibitors.

Even fewer complications should be expected from drugs that inhibit receptor binding of pro-tumorigenic cytokines or chemokines. Several anti-cytokine drugs are already in use for the treatment of chronic inflammatory diseases or are under clinical development for such usage. Although cytokine inhibitors alone are unlikely to cause cancer cell death, several phase I/II clinical trials currently evaluate the efficacy of anti-IL-6 and anti-TNF- α drugs as single agents in various cancers (Balkwill, 2009). The effects obtained so far include disease stabilization and partial responses, but by-and-large the therapeutic effects are modest and underscore the necessity of evaluating such drugs in combination with conventional therapy. Anti-chemokine drugs are also being evaluated, including receptor antagonists and blocking antibodies, targeting CCR2, CCR4, and CXCR4 (Balkwill, 2009). IL-1 inhibition in multiple myeloma slows tumor growth and leads to a chronic disease state, thereby preventing progression to active myeloma (Lust et al., 2009).

Metastasis presents another important application and challenge for drugs that target tumor-associated inflammation. Recently, an anti-RANKL antibody, which was developed for the treatment of osteoporosis, has been found effective in inhibition of bone metastasis in prostate cancer (Hurst et al., 2009). Other experiments done in mice have shown that NF- κ B inhibition in metastatic cancer cells or neutralization of TNF- α can convert inflammation promoted metastatic growth to inflammation-induced tumor regression, dependent on IFN-induced TRAIL expression (Luo et al., 2004). Such findings illustrate how manipulation of cytokine expression can be used to convert tumor- and metastasis-promoting inflammation to a strong anti-tumor response.

Conclusions and Prospective

Inflammation can affect every aspect of tumor development and progression as well as the response to therapy. In the past 10 years, we have learned a great deal about the different mechanisms by which cancer and inflammation intersect, and the time is right to translate much of the basic knowledge gained thus far and use it to add new armaments to the arsenal of cancer therapeutics. Only by targeting every single aspect of cancer biology, can we expect to make real gains in the fight against these currently incurable diseases. In addition to a combination of anti-inflammatory approaches that target the tumor microenvironment with more sophisticated and selective tumoricidal drugs, future therapies should also take notice of the natural genetic variation that affects inflammation and immunity. Such considerations are extremely important in the design of new preventive approaches to the reduction of cancer risk that need to be applied to large populations composed of relatively healthy individuals. Indeed, one of the major lessons learned from investigating the relationships between inflammation and cancer, is that most cancers are preventable. Prevention is a much better and more economic way to fight cancer than treating an already advanced and often intractable disease, as is done at the present.

Text Box: Inflammation and cancer-basic facts

1. Chronic inflammation increases cancer risk.
2. Subclinical, often undetectable, inflammation may be as important in increasing cancer risk (for instance, obesity-induced inflammation).
3. Various types of immune and inflammatory cells are frequently present within tumors.
4. Immune cells affect malignant cells through production of cytokines, chemokines, growth factors, prostaglandins and reactive oxygen and nitrogen species.
5. Inflammation impacts every single step of tumorigenesis, from initiation through tumor promotion, all the way to metastatic progression.
6. In developing tumors anti-tumorigenic and pro-tumorigenic immune and inflammatory mechanisms coexist, but if the tumor is not rejected, the pro-tumorigenic effect dominates.
7. Signaling pathways that mediate the pro-tumorigenic effects of inflammation are often subject to a feed-forward loop (for example, activation of NF- κ B in immune cells induces production of cytokines that activate NF- κ B in cancer cells to induce chemokines that attract more inflammatory cells into the tumor).
8. Certain immune and inflammatory components may be dispensable during one stage of tumorigenesis but absolutely critical in another stage.

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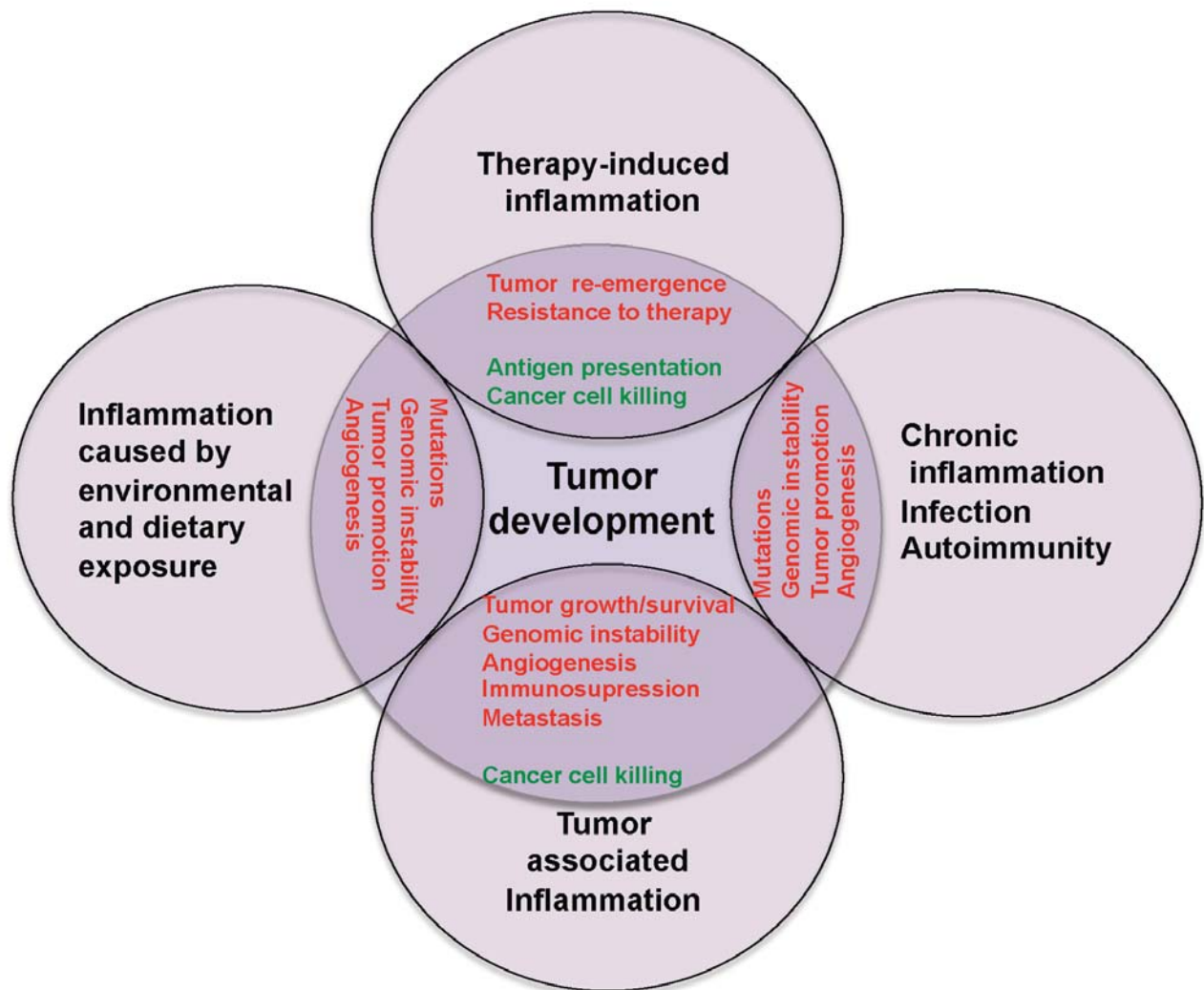
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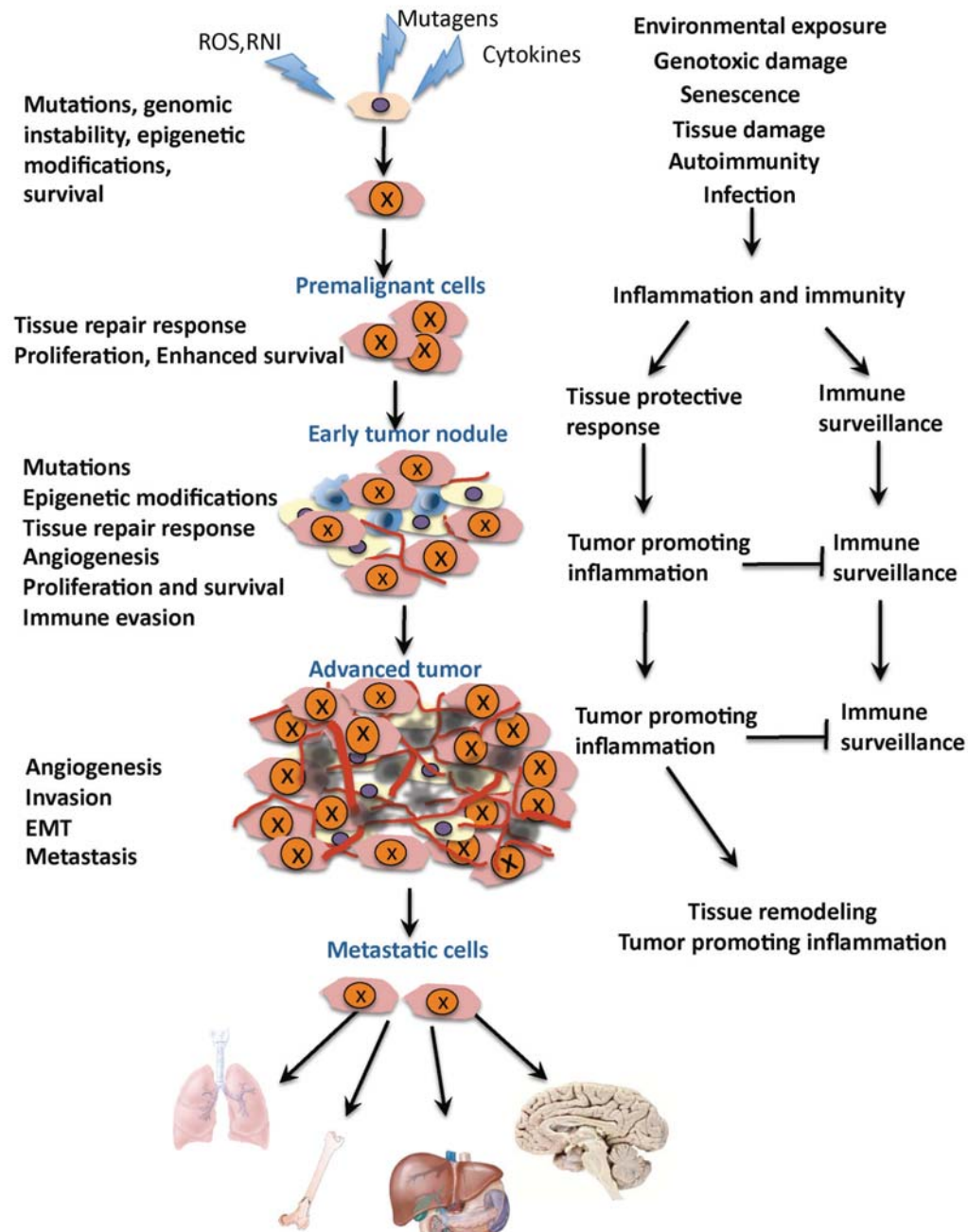
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**Figure 1.**

Types of inflammation in tumorigenesis and cancer.

Chronic inflammation associated with infections or autoimmune disease precedes tumor development and can contribute to it through induction of oncogenic mutations, genomic instability, early tumor promotion, and enhanced angiogenesis. Prolonged exposure to environmental irritants or obesity can also result in low-grade chronic inflammation that precedes tumor development and contributes to it through the mechanisms mentioned above. Tumor-associated inflammation goes hand in hand with tumor development. This inflammatory response can enhance neo-angiogenesis, promote tumor progression and metastatic spread, cause local immunosuppression, and further augment genomic instability. Cancer therapy can also trigger an inflammatory response by causing trauma, necrosis, and tissue injury that stimulate tumor re-emergence and resistance to therapy. However, in some cases, therapy-induced inflammation can enhance antigen presentation, leading to immune-mediated tumor eradication. Tumor promoting mechanisms are in red and anti-tumorigenic mechanisms are in green.

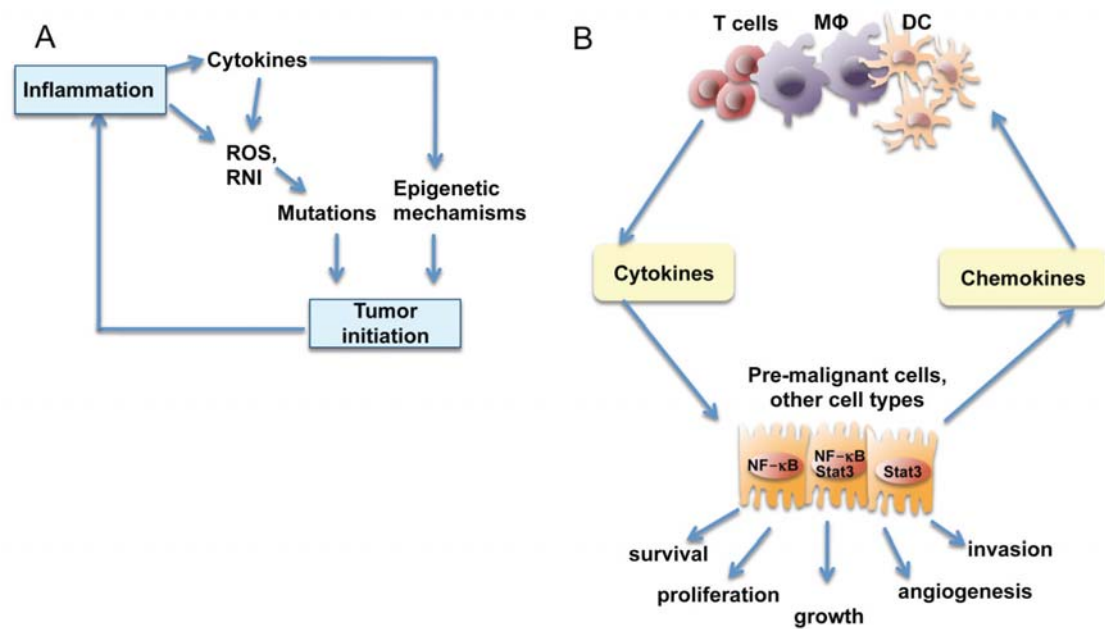
**Figure 2.**

The multifaceted role of inflammation in cancer

Inflammation acts at all stages of tumorigenesis. It may contribute to tumor initiation through mutations, genomic instability, and epigenetic modifications. Inflammation activates tissue repair responses, induces proliferation of premalignant cells, and enhances their survival.

Inflammation also stimulates angiogenesis, causes localized immunosuppression, and promotes the formation of a hospitable microenvironment in which pre-malignant cells can survive, expand, and accumulate additional mutations and epigenetic changes. Eventually, inflammation also promotes metastatic spread. Mutated cells are marked with "X". Yellow - stromal cells, Brown - malignant cells, Red - blood vessels, Blue - immune and inflammatory

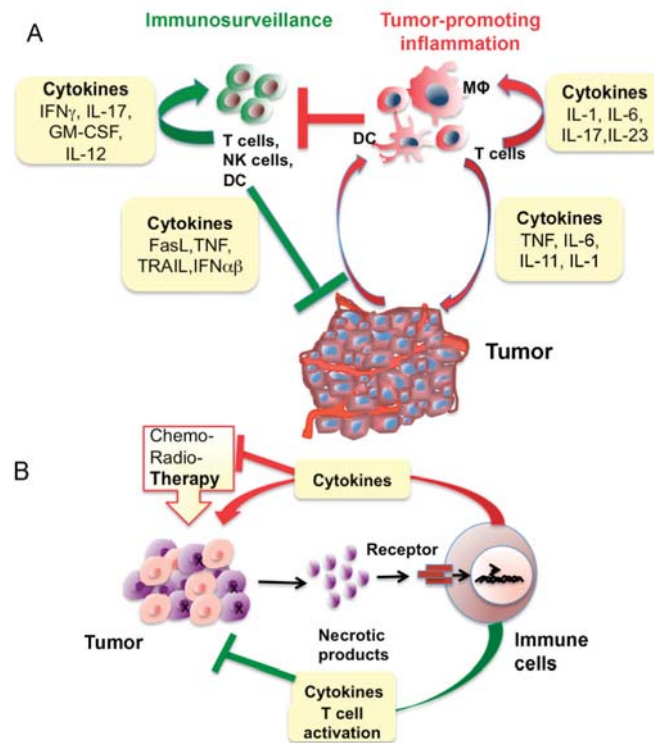
cells. Epithelial-mesenchymal transition, EMT; reactive oxygen species, ROS; reactive nitrogen intermediates (RNI)

**Figure 3.**

Role of inflammation in tumor initiation and promotion

A) Tumor initiation. Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) produced by inflammatory cells may cause mutations in neighboring epithelial cells. Also, cytokines produced by inflammatory cells can elevate intracellular ROS and RNI in pre-malignant cells. In addition, inflammation can result in epigenetic changes that favor tumor initiation. Tumor-associated inflammation contributes to further ROS, RNI and cytokine production.

B) Tumor promotion. Cytokines produced by tumor infiltrating immune cells activate key transcription factors, such as NF-κB or STAT3, in pre-malignant cells to control numerous pro-tumorigenic processes, including survival, proliferation, growth, angiogenesis, and invasion. As parts of positive feed-forward loops, NF-κB and STAT3 induce production of chemokines that attract additional immune/inflammatory cells to sustain tumor-associated inflammation.

**Figure 4.**

Immunosurveillance, tumor-promoting and therapy-induced inflammation.

A) Balance between immunosurveillance and tumor promoting inflammation in the tumor microenvironment. Tumor promoting cytokines act on immune and malignant cells to tilt the balance toward tumor promotion. Tumor promoting immunity dampens immunosurveillance, which otherwise inhibits tumor growth. B) Therapy-induced inflammation. Various forms of therapy induce death (necrosis) of malignant cells resulting in the release of necrotic products and damage-associated molecular patterns (DAMPs) that activate cytokine-producing inflammatory cells. These cytokines activate pro-survival genes in residual cancer cells, rendering them resistant to subsequent rounds of therapy. However, in some cases, therapy-induced inflammation augments the presentation of tumor antigens and stimulates an anti-tumor immune response that improves the therapeutic outcome.

Table 1

Roles of different subtypes of immune and inflammatory cells in anti-tumor immunity and tumor-promoting inflammation

Cell types	Anti-tumor	Tumor-promoting
Macrophages, dendritic cells, myeloid-derived suppressor cells	Antigen presentation Production of cytokines (IL-12 and type I IFN)	Immunosuppression Production of cytokines, chemokines, proteases, growth factors, and angiogenic factors
Mast cells		Production of cytokines
B cells	Production of tumor specific antibodies?	Production of cytokines Activation of mast cells Immunosuppression
CD8 ⁺ T cells	Direct lysis of cancer cells Production of cytotoxic cytokines	Production of cytokines?
CD4 ⁺ Th2 cells		Education of macrophages Production of cytokines B cell activation
CD4 ⁺ Th1 cells	Help to cytotoxic T lymphocytes (CTLs) in tumor rejection	Production of cytokines
	Production of cytokines (IFN γ)	
CD4 ⁺ Th17 cells	Activation of CTLs	Production of cytokines
CD4 ⁺ Treg cells	Suppression of inflammation (cytokines and other suppressive mechanisms)	Immunosuppression Production of cytokines
Natural Killer cells	Direct cytotoxicity toward cancer cells Production of cytotoxic cytokines	
Natural Killer T cells	Direct cytotoxicity toward cancer cells Production of cytotoxic cytokines	
Neutrophils	Direct cytotoxicity Regulation of CTL responses	Production of cytokines, proteases, and ROS

Exhibit 103



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Oxidative stress, inflammation, and cancer: How are they linked?

Simone Reuter, Subash C. Gupta, Madan M. Chaturvedi, and Bharat B. Aggarwal

Cytokine Research Laboratory, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA

Abstract

Extensive research during last two decades has revealed the mechanism by which continued oxidative stress can lead to chronic inflammation, which in turn could mediate most chronic diseases including cancer, diabetes, cardiovascular, neurological and pulmonary diseases. Oxidative stress can activate a variety of transcription factors including NF- κ B, AP-1, p53, HIF-1 α , PPAR- γ , β -catenin/Wnt, and Nrf2. Activation of these transcription factors can lead to the expression of over 500 different genes, including those for growth factors, inflammatory cytokines, chemokines, cell cycle regulatory molecules, and anti-inflammatory molecules. How oxidative stress activates inflammatory pathways leading to transformation of a normal cell to tumor cell, tumor cell survival, proliferation, chemoresistance, radioresistance, invasion, angiogenesis and stem cell survival is the focus of this review. Overall, observations to date suggest that oxidative stress, chronic inflammation, and cancer are closely linked.

Keywords

Oxidative stress; Inflammation; Cancer; Pro-oxidants; Anti-oxidants; NF- κ B

1. Introduction

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), and their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism [1]. ROS are products of a normal cellular metabolism and play vital roles in stimulation of signaling pathways in plant and animal cells in response to changes of intra- and extracellular environmental conditions [2]. Most ROS are generated in cells by the mitochondrial respiratory chain [3]. During endogenous metabolic reactions, aerobic cells produce ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^\bullet), and organic peroxides as normal products of the biological reduction of molecular oxygen [4]. The electron transfer to molecular oxygen occurs at the level of the respiratory chain, and

Address correspondence to: Bharat B. Aggarwal, Ph.D., Cytokine Research Laboratory, Department of Experimental Therapeutics (Unit 0143), The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA, aggarwal@mdanderson.org; Tel: +1 713-794-1817; Fax: +1-713-745-6339.

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the electron transport chains are located in membranes of the mitochondria [5,6]. Under hypoxic conditions, the mitochondrial respiratory chain also produces nitric oxide (NO), which can generate other reactive nitrogen species (RNS) [3]. RNS can further generate other reactive species, e.g., reactive aldehydes-malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), by inducing excessive lipid peroxidation [7]. Proteins and lipids are also significant targets for oxidative attack, and modification of these molecules can increase the risk of mutagenesis [8].

Under a sustained environmental stress, ROS are produced over a long time, and thus significant damage may occur to cell structure and functions and may induce somatic mutations and neoplastic transformation [9,10]. Indeed, cancer initiation and progression has been linked to oxidative stress by increasing DNA mutations or inducing DNA damage, genome instability, and cell proliferation [11].

The skin, for example, is chronically exposed to both endogenous and environmental pro-oxidants due to its interface function between the body and the environment, and to protect the skin against this overload of oxidant species, it needs a well-organized system of both chemical and enzymatic antioxidants [12]. The lungs, which are directly exposed to oxygen concentrations higher than in most other tissues, are protected against these oxidants by a variety of antioxidant mechanisms [13]. Furthermore, aging, which is considered as an impairment of body functions over time, caused by the accumulation of molecular damage in DNA, proteins and lipids, is also characterized by an increase in intracellular oxidative stress due to the progressive decrease of the intracellular ROS scavenging [14]. Acting to protect the organism against these harmful pro-oxidants is a complex system of enzymatic antioxidants [e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, catalase] and nonenzymatic antioxidants [e.g., glutathione (GSH), vitamins C and D] [15] (Figure 1).

ROS are involved in a wide spectrum of diseases, including chronic inflammation (Table 1), and in a wide variety of different cancers (Table 2).

Chronic inflammation is induced by biological, chemical, and physical factors and is in turn associated with an increased risk of several human cancers [54]. The link between inflammation and cancer has been suggested by epidemiological and experimental data [55,56] and confirmed by anti-inflammatory therapies that show efficacy in cancer prevention and treatment [57]. The fact that continuous irritation over long periods of time can lead to cancer had already been described in the traditional Ayurvedic (meaning, the science of long life) medical system, written as far back as 5000 years ago [58]. Whether this irritation is the same as what Rudolf Virchow referred to as inflammation in the nineteenth century is uncertain [59]. Virchow first noted that inflammatory cells are present within tumors and that tumors arise at sites of chronic inflammation [60]. This inflammation is now regarded as a “secret killer” for diseases such as cancer. For example, inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are associated with increased risk of colon adenocarcinoma [61-63], and chronic pancreatitis is related to an increased rate of pancreatic cancer [64].

The exact mechanisms by which a wound-healing process turns into cancer are topics of intense research [57,65], and possible mechanisms include induction of genomic instability, alterations in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation of initiated cells, resistance to apoptosis, aggressive tumor neo-vascularization, invasion through tumor-associated basement membrane, and metastasis [66]. How oxidative stress modulates these different stages of inflammation-induced carcinogenesis is the focus of this review.

2. Inflammatory network

The sources of inflammation are widespread and include microbial and viral infections, exposure to allergens, radiation and toxic chemicals, autoimmune and chronic diseases, obesity, consumption of alcohol, tobacco use, and a high-calorie diet [60,67]. In general, the longer the inflammation persists, the higher the risk of cancer. Two stages of inflammation exist, acute and chronic inflammation. Acute inflammation is an initial stage of inflammation (innate immunity), which is mediated through the activation of the immune system. This type of inflammation persists only for a short time and is usually beneficial for the host. If the inflammation lasts for a longer period of time, the second stage of inflammation, or chronic inflammation, sets in and may predispose the host to various chronic illnesses, including cancer [68]. During inflammation, mast cells and leukocytes are recruited to the site of damage, which leads to a 'respiratory burst' due to an increased uptake of oxygen, and thus, an increased release and accumulation of ROS at the site of damage [7,65].

On the other hand, inflammatory cells also produce soluble mediators, such as metabolites of arachidonic acid, cytokines and chemokines, which act by further recruiting inflammatory cells to the site of damage and producing more reactive species. These key mediators can activate signal transduction cascades as well as induce changes in transcription factors, such as nuclear factor kappa B (NF- κ B), signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor-1 α (HIF1- α), activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and NF-E2 related factor-2 (Nrf2), which mediate immediate cellular stress responses (Figure 2). Induction of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), aberrant expression of inflammatory cytokines [tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6 and chemokines [IL-8; CXC chemokine receptor 4 (CXCR4)], as well as alterations in the expression of specific microRNAs, have also been reported to play a role in oxidative stress-induced inflammation [69]. This sustained inflammatory/oxidative environment leads to a vicious circle, which can damage healthy neighboring epithelial and stromal cells and over a long period of time may lead to carcinogenesis [70].

As an example, mutations in the rat sarcoma viral oncogene (RAS) induce an inflammatory response. RAS, which is mutated in approximately 25% of all malignancies [71], promotes cell proliferation, tumor growth, and angiogenesis of malignant cells. During inflammatory stimuli, Ras induces the expression of various inflammatory gene products, including the pro-inflammatory cytokines IL-1, IL-6 and IL-11 and the chemokine IL-8 [72].

3. Pro-oxidant network

Following an inflammatory stimulus, initiation of carcinogenesis mediated by ROS may be direct (oxidation, nitration, halogenation of nuclear DNA, RNA, and lipids), or mediated by the signaling pathways activated by ROS. With the help of the mitochondrial respiratory chain, aerobic organisms are able to attain a far greater energy production efficiency compared with anaerobic organisms. However, one disadvantage of aerobic respiration is continuous electron leakage to O₂ during mitochondrial ATP synthesis. In fact, 1–5% of total oxygen consumed in aerobic metabolism gives rise to the superoxide anion (O₂⁻), an example of ROS. To protect against this free radical, the main enzyme for its degradation, the manganese-superoxide dismutase (Mn-SOD), dismutates it into H₂O₂ and water [73].

H₂O₂, another example of ROS, may be formed either by dismutation from superoxide anion or spontaneously in peroxisomes from molecular oxygen [74-76]. Despite its lesser reactivity compared with other ROS, H₂O₂ plays however an important role in carcinogenesis because it is capable of diffusing throughout the mitochondria and across cell

membranes and producing many types of cellular injury [74,75]. The main injurious effects of ROS in mammalian cells are however mediated by the hydroxyl radical ($\cdot\text{OH}$). It has a very unstable electron structure and is therefore unable to diffuse more than one or two molecular diameters before it reacts in practice with any cellular component [76,77]. The majority of $\cdot\text{OH}$ in vivo is produced in the presence of reduced transition metals (ions of Fe, Cu, Co, or Ni), mainly via the Fenton reaction when Fe^{2+} contacts H_2O_2 . The $\cdot\text{OH}$ -derived DNA damage includes the generation of 8-hydroxyguanosine (8-OHG), the hydrolysis product of which is 8-hydroxydeoxyguanosine (8-OHdG). 8-OHdG is the most widely used fingerprint of radical attack towards DNA [77,78]. 8-OHdG has been strongly implicated in carcinogenesis progression. For example, in breast carcinomas, 8-OHdG has been reported to be increased 8- to 17-fold in breast primary tumors compared with nonmalignant breast tissue [79-81].

$\text{NO}\cdot$, another free radical implicated in carcinogenesis, is a short-lived free radical generated from L-arginine [82], that is effective against pathogens. The major part of $\text{NO}\cdot$ is synthesized by iNOS, usually after challenge by immunological or inflammatory stimuli [82,83]. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). The constitutive (calcium-dependent) isoforms, neuronal NOS (nNOS or bNOS) and endothelial NOS (eNOS), produce small amounts of NO which act as a neurotransmitter and vasodilator, respectively [84]. The inducible (calcium-independent) isoform (iNOS) produces much larger amounts of NO and is only expressed during inflammation. Whereas iNOS can produce injurious amounts of RNS (check), eNOS and nNOS produce beneficial amounts under physiological conditions [85]. iNOS is induced by cytokines such as γ -interferon (γ -IFN), TNF- α , IL-1, and lipopolysaccharide (LPS). LPS activation induces the translocation of NF- κB , from the cytoplasm to the nucleus, where it interacts with κB elements in the *NOS2* (*iNOS*) 5' flanking region, triggering *NOS2* transcription [86].

Defective autophagy of old mitochondria (mitophagy) can also be a major source of ROS [87]. These ROS produced by damaged mitochondria, can promote tumor development, likely by perturbing the signal transduction adaptor function of p62-controlling pathways [88].

To control the balance between production and removal of ROS (Figure 3), a variety of DNA repair enzymes exist, although antioxidants are more specific and efficient in protecting cells from radicals. This antioxidant system includes both endogenous and exogenous and enzymatic and non-enzymatic antioxidants. Glutathione (GSH), is a tripeptide and the major endogenous antioxidant produced by the cells, which helps to protect cells from ROS such as free radicals and peroxides [89]. It is now well established that ROS and electrophilic chemicals can damage DNA, and that GSH can protect against this type of damage [90]. GSH can also directly detoxify carcinogens through phase II metabolism and subsequent export of these chemicals from the cell. On the other hand, elevated GSH levels are observed in various types of cancerous cells and solid tumors, and this tends to make these cells and tissues more resistant to chemotherapy [91-93].

SODs were the first characterized antioxidant enzymes [94]. Three different types of SOD are expressed in human cells, copper-zinc SOD (Cu-ZnSOD), Mn-SOD, and extracellular-SOD (EC-SOD), all of which are able to dismutate two $\text{O}_2^{\cdot -}$ anions to H_2O_2 and molecular oxygen. Catalase is then responsible for detoxification of H_2O_2 to water. GPx are another group of enzymes capable of reducing hydroperoxides, including lipid hydroperoxides, using GSH as substrate. The oxidized form of glutathione disulfide (GSSG) is again reduced by the specific enzyme glutathione reductase. Peroxiredoxins (Prx) were first described 20 years ago and as in catalase and GPx, the main function of peroxiredoxins is to reduce alkyl hydroperoxides and H_2O_2 to the corresponding alcohol or water.

Direct effects of ROS, generally attributed to high concentrations at the site of damage, include DNA strand breaks, point mutations, aberrant DNA cross-linking, and mutations in proto-oncogenes and tumor-suppressor genes, thus promoting neoplastic transformation [7,95]. For example, ROS can reduce the expression and enzymatic activity of the DNA mismatch repair genes mutS homologue 2 and 6 and can increase the expression of DNA methyltransferases, leading to a global hypermethylation of the genome [60]. This leads to promoter silencing of several genes, such as adenomatous polyposis coli (APC), cyclin-dependent kinase inhibitor-2 (CDKN-2), breast cancer susceptibility gene 1 (BRCA1), retinoblastoma protein (Rb), and murine double minute 2 (MDM2), and the DNA mismatch repair gene, human mutL homolog 1 (hMLH1) [96,97].

On the other hand, low or transient levels of ROS can activate cellular proliferation or survival signaling pathways, such as the NF- κ B, AP1, extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), and phosphoinositide 3-kinase/AKT8 virus oncogene cellular homolog (PI3K/Akt) pathways (Table 3).

For example, H₂O₂ is able to degrade I κ B α , the inhibitory subunit of NF- κ B [137]. Protein kinase C, which participates in a variety of pathways regulating transcription and cell cycle control, is also activated by H₂O₂ [137]. In addition, ROS induces both the activation and synthesis of AP-1, a regulator of cell growth, proliferation, and apoptosis [138,139] and transcription factors such as STAT3, HIF-1 α , and p53 [118,140,141].

4a. Cellular transformation

Chronic inflammation has been linked to various steps involved in carcinogenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis [65,142]. How oxidative stress is involved in these various steps is discussed in the following sections.

Cancer is a multistage process defined by at least three stages: initiation, promotion, and progression [143-145]. Oxidative stress interacts with all three stages of this process. During the initiation stage, ROS may produce DNA damage by introducing gene mutations and structural alterations of the DNA. In the promotion stage, ROS can contribute to abnormal gene expression, blockage of cell- to cell communication, and modification of second messenger systems, thus resulting in an increase of cell proliferation or a decrease in apoptosis of the initiated cell population. Finally, oxidative stress may also participate in the progression stage of the cancer process by adding further DNA alterations to the initiated cell population [146].

In recent years, considerable evidence has demonstrated that ROS are involved in the link between chronic inflammation and cancer [147-149]. Indeed, an important characteristic of tumor promoters is their ability to recruit inflammatory cells and to stimulate them to generate ROS [150,151]. Tumor promotion, for example, can be inhibited in animal models by the use of agents, including certain antioxidants as well as steroids and retinoids, that can inhibit the phagocyte respiratory burst [148,150]. Moreover, increased levels of oxidatively modified DNA bases (such as thymidine glycol, 5-hydroxymethyl-2'-deoxyuridine and 8-OHdG) have been induced in the skin of mice by topical phorbol 12-myristate 13- acetate (PMA) exposure [152]. 8-OHdG has also been identified in the epidermis of nude mice exposed to near-UV [153]. In addition, genetic damage and neoplastic transformation have been demonstrated in cells co-cultured in vitro with activated phagocytes [149] and the genotoxic effects observed include formation of DNA strand breaks [151], sister chromatid exchange [154] and mutations [155]. Furthermore, the DNA base modifications observed are characteristic of an attack by reactive oxygen species OH \cdot . [156]. Inflammatory cells may also increase DNA damage by activating procarcinogens to DNA-damaging species, for

example neutrophils can activate aromatic amines, aflatoxins, estrogens, phenols, and polycyclic aromatic hydrocarbons by ROS-dependent mechanisms [148,157]. On the other hand, both neutrophils and macrophages have themselves been shown to release large quantities of superoxide, hydrogen peroxide, and hydroxyl radical following activation of their redox metabolism [158].

In fact, initial experiments on the role of ROS in tumor initiation have assumed that oxidative stress acts as a DNA-damaging agent, effectively increasing the mutation rate within cells and thus promoting oncogenic transformation [159]. However, more recent studies have revealed that in addition to inducing genomic instability, ROS can specifically activate certain signaling pathways and thus contribute to tumor development through the regulation of cellular proliferation, angiogenesis, and metastasis [160]. For example, nitrosative stress has been shown to play a critical role in inflammation-associated carcinogenesis by activating AP-1, a representative redox-sensitive transcription factor [161], which is involved in cell transformation and proliferation [139,162].

4b. Tumor cell survival

One of the key characteristics of tumor cells is their increased ability to survive compared with normal cells. ROS are reported to be tumorigenic by virtue of their ability to increase cell proliferation, survival, and cellular migration. ROS can induce DNA damage, leading to genetic lesions that initiate tumorigenicity and subsequent tumor progression. On the other hand, ROS can also induce cellular senescence and cell death and can therefore function as anti-tumorigenic agents. Whether ROS promote tumor cell survival or act as anti-tumorigenic agents depends on the cell and tissues, the location of ROS production, and the concentration of individual ROS.

ROS has been reported to play a major role in tumor initiation and survival induced by a variety of agents both in animal models and humans [158,163,164] by mediating cellular signal transduction pathways. These signaling pathways are involved in the transmission of inter or intracellular information and are critical for supporting tumor cell survival and establishing cell fate. The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) family of enzymes, one of the potential sources of ROS production, has been reported to promote tumor cell survival and growth [165]. For example, Nox4 and Nox5 promote tumor cell survival in pancreatic and lung cancers, respectively [165]. The serine-threonine kinase Akt has been reported to down-regulate antioxidant defenses and promote tumor cell survival [166]. ROS has also been reported to activate Akt by inhibiting phosphatase and tensin homolog deleted from chromosome 10 (PTEN), the phosphatase counteracting PI3K-dependent Akt activation [167]. Akt may foster tumorigenesis by multiple means [168,169], for example, by stabilizing cellular avian myeloblastosis virus oncogene (c-Myc) and cyclin D1 or by inducing degradation of the cyclin-dependent kinase (Cdk) inhibitor, p27 kinase inhibitor protein (p27Kip1). Akt is also a profound inhibitor of apoptosis due to its ability to inactivate pro-apoptotic molecules, including caspase-9 and the Bcl-2 homology3 (BH3)-only protein Bcl-XL/Bcl-2-associated death promoter (Bad), and by triggering the activity of the transcription factor NF- κ B. In addition, Akt promotes nuclear translocation of the ubiquitin ligase MDM2, which counteracts p53-mediated apoptosis. An important aspect of Akt's promotion of cell survival involves alterations in cellular energy metabolism [168,169]. Thus, by preventing apoptosis and increasing oxidative metabolism, Akt lies at the hub of complex signaling networks that integrate a multitude of potentially oncogenic signals.

4c. Tumor cell proliferation

Uncontrolled tumor cell proliferation requires the upregulation of multiple intracellular signaling pathways including cascades involved in survival, proliferation, and cell cycle progression. The most significant effects of oxidants on signaling pathways have been observed in the mitogen-activated protein (MAP) kinase/AP-1 and NF- κ B pathways [170]. The induction of redox-sensitive pathways during tumor cell proliferation is necessary since cell division presents tremendous energy requirements and the production of metabolites from energy-generating reactions must be buffered to prevent oxidative damage and ultimately cell death [171].

Of the MAP kinase family, which modulates gene expression through phosphorylation of a wide array of transcription factors, the ERK pathway is the most commonly linked with the regulation of cell proliferation. Activation of the ERK, c-Jun N-terminal kinase (JNK), and p38 subfamilies has been observed in response to changes in the cellular redox balance [172]. The induction of AP-1 by H₂O₂, cytokines, and other stressors, for example, is mediated mainly by JNK and p38 MAP kinase cascades [173]. Once activated, JNK proteins translocate to the nucleus and phosphorylate c-Jun and activating transcription factor-2 (ATF-2), enhancing transcriptional activities [174,175]. H₂O₂ can activate MAP kinases and thereby AP-1 in several manners.

Redox status has also been shown to have an impact on NF- κ B regulation. NF- κ B regulates several genes involved in cell transformation, proliferation, and angiogenesis [176]. Carcinogens and tumor promoters including UV radiation, phorbol esters, asbestos, alcohol, and benzo(a)pyrene are among the external stimuli that activate NF- κ B [177,178]. Expression of NF- κ B has been shown to promote cell proliferation, whereas inhibition of NF- κ B activation blocks cell proliferation [179]. Additionally, tumor cells from blood neoplasms, and cell lines from different cancers, including colon, breast, pancreas, and squamous cell carcinoma, have all been reported to constitutively express activated NF- κ B [180]. The mechanism for activation of NF- κ B by ROS is not clear, and the relationship between NF- κ B and ROS is complex [123]. Although mild oxidative stress can lead to modest NF- κ B activation, extensive oxidative stress can inhibit NF- κ B [123]. Furthermore, NF- κ B can protect cells from oxidative stress through induction of the ferritin heavy chain and SOD2 genes, which are both regulated by NF- κ B [181,182]. On the other hand, ROS are believed to be implicated as second messengers involved in activation of NF- κ B via TNF and IL-1 [183] and indeed, suppression of TNF and IL-1 were shown to downregulate the expression of active NF- κ B and inhibit proliferation of lymphoma and myelogenous leukemia cells [184]. The importance of ROS on NF- κ B activation is further supported by studies demonstrating that activation of NF- κ B by nearly all stimuli can be blocked by antioxidants, such as L-cysteine, N-acetylcysteine (NAC), thiols, green tea polyphenols, and vitamin E [185,186], although this might be not very specific because antioxidants have multiple targets [187]. Likewise, NF- κ B activity was increased in cells that overexpressed SOD and decreased in cells overexpressing catalase [188].

Kinases, such as protein kinase C (PKC) can also be activated by H₂O₂ and redox cycling quinones [189,190]. Similarly, H₂O₂ leads to the activation of protein kinase B/Akt (PKB/Akt), which is associated with heat shock protein 27 (Hsp27) [191].

That ROS such as H₂O₂ and superoxide anion induce mitogenesis and cell proliferation has now been demonstrated in several mammalian cell types [192]; and a reduction in cellular oxidants via supplementation with antioxidants such as superoxide dismutase, catalase, β -carotene, and flavonoids inhibits cell proliferation in vitro [193]. However, paradoxically high concentrations of ROS can trigger apoptotic or necrotic cell death [194-196].

4d. Tumor cell invasion

Oxygen radicals may augment tumor invasion and metastasis by increasing the rates of cell migration. During transformation into invasive carcinoma, epithelial cells undergo profound alterations in morphology and adhesive mode, resulting in a loss of normal epithelial polarization and differentiation, and a switch to a more motile, invasive phenotype. For example, treatment of mammalian carcinoma cells with hydrogen peroxide prior to intravenous injection into mice enhances lung metastasis formation, indicating that an important function for ROS is the seeding of metastatic tumor cells [197]. This might be due to a decreased attachment of tumor cells to the basal lamina, or alternatively be due to the increased activity or expression of proteins that regulate cellular motility. For instance, oxidative stress regulates the expression of intercellular adhesion protein-1 (ICAM-1), a cell surface protein in endothelial and epithelial cells, most likely due to the activation of NF- κ B. ICAM-1 together with IL-8 regulates the transendothelial migration of neutrophils and has a potential function in tumor metastasis [198].

On the other hand, it is believed that the matrix metalloproteinases (MMPs) play the central role, and their increased expression reportedly is associated with the invasion and metastasis of malignant tumors of different histogenetic origins [199]. For example, Mori et al. found that MMP-13, MMP-3, and MMP-10 were remarkably upregulated by the oxidant directly, and their activities were critically implicated in the invasive potential induced in NMuMG cells in the reconstituted model [200]. Another subgroup of MMPs, gelatinases (MMP-2 and -9), which are key enzymes for degrading type IV collagen and are thought to play a critical role in tumor invasion and metastasis [199], were also found to be activated post-transcriptionally by prolonged oxidative treatment. These effector molecules activated under prolonged oxidative stress relate chronic inflammation to malignant transformation, in particular to the invasive potential of cells, at least at a molecular level.

MMPs are capable of cleaving most components of the basement membrane and extracellular matrix [201]. The activation of MMPs, such as MMP-2, probably occurs by the reaction of ROS with thiol groups in the protease catalytic domain [202]. In addition to their role as key regulators of MMP activation, ROS have been implicated in MMP gene expression [203]. Both hydrogen peroxide and nitric oxide donors, as well as the increased expression of iNOS, stimulate the expression of several MMPs (MMP-1, MMP-3, MMP-9, MMP-10, MMP-13) [203]. In fibroblastic cells, the sustained production of H₂O₂ recently was shown to activate MMP-2 and to increase cell invasion [204]. Oxidative stress may also modulate MMP expression by activation of the rat sarcoma viral oncogene (RAS), or direct activation of the MAPK family members extracellular-signal regulated kinase 1/2 (ERK1/2), p38, and JNK, or inactivation of phosphatases that regulate these proteins [160].

In addition, several studies have reported the involvement of chemokines and chemokine receptors in the invasion and metastasis of different types of tumors [205-208]. The metastatic potential of chemokines is attributed to their ability to induce the expression of MMPs, which facilitate tumor invasion [208,209]. Moreover, silencing of endogenous CXCR4 gene expression by CXCR4-shRNA inhibited the proliferation, adhesion, chemotaxis and invasion of mucoepidermoid carcinoma cells [210]. In addition, recent data point to a role for the small guanosine triphosphatase Rac1 (GTPase Rac1) in motility and invasion of tumor cells in vitro by altering cell-cell and cell-matrix adhesion. For example, Rac1 activity induces ROS production in endothelial cells. These ROS can mediate Rac1-induced loss of cell-cell adhesion in primary human endothelial cells and thus might loosen the integrity of the endothelium [211].

It is becoming clear that a number of steps in the metastatic cascade, such as invasion, intravasation and extravasation are regulated by redox signaling [212]. One such redox

signalling molecule is the electrophilic cyclopentenone prostaglandin 15d-PGJ2 (15-deoxy-12,14-prostaglandin J2), an inflammatory molecule [213], that can affect redox signalling through the post-translational modification of critical cysteine residues in proteins, such as actin, vimentin and tubulin [214,215]. The fact that 15d-PGJ2 can alter the cytoskeleton [212], may coincide with decreased migration and increased focal-adhesion disassembly, that might have important implications in the inhibition of metastatic processes such as invasion, intravasation and extravasation. These results suggest a role for redox signalling pathways, rather than direct cytoskeletal disruption, in the mechanism of 15d-PGJ2 in cancer cells.

Finally, Cheng et al demonstrated that ROS enhance the transendothelial migration (TEM) of melanoma cells during intravasation, and that this mechanism could potentially be triggered by ultraviolet radiation through the increased expression of thioredoxin interacting protein (Txnip) and inhibition of thioredoxin (Trx) [216].

4e. Tumor cell angiogenesis

Solid tumors induce an angiogenic response by the host blood vessels to form a new vascular network for the supply of nutrients and oxygen [217]. This neovascular response is partly responsible for tumor growth and metastatic spread [218,219]. Angiogenesis in tumors is controlled by the so-called 'angiogenic switch,' which allows the transition from low invasive and poorly vascularized tumors to highly invasive and angiogenic tumors. To further increase in size, tumor cells express a set of molecules that initiate tumor vascularization.

A number of cellular stress factors, including hypoxia, nutrient deprivation, and ROS, are important stimuli of angiogenic signaling [220]. In addition, overexpression of Ras has been linked to vascularization of tumors [221]. Indeed, transformation by Ras stabilizes HIF-1 α and upregulates the transcription of vascular endothelial growth factor-A (VEGF-A). Moreover, chemical antioxidants inhibit the mitogenic activity of Ras, indicating that ROS participate directly in malignant transformation. Finally, ROS stabilize HIF-1 α protein and induce production of angiogenic factors by tumor cells [222].

The HIF system plays a significant role in angiogenesis, and the molecular mechanisms of its regulation have recently been characterized. In addition, HIF-independent mechanisms that involve a number of other molecules and transcription factors such as NF- κ B and p53 have been described. p53 may interact with the HIF system but may also have direct effects on angiogenesis regulators or interfere with translation mechanisms of angiogenesis factors

One other major factor in angiogenesis is vascular endothelial growth factor (VEGF), which is produced by the cells to stimulate the growth of new blood vessels. VEGF induces angiogenesis by stimulating endothelial cell proliferation and migration primarily through the receptor tyrosine kinase VEGF receptor2, fetal liver kinase 1/kinase insert domain receptor (Flk1/KDR). VEGF binding initiates tyrosine phosphorylation of KDR, which results in activation of downstream signaling enzymes including ERK1/2, Akt and endothelial nitric oxide synthase (eNOS), which contribute to angiogenic-related responses in endothelial cells [134]. A number of oncogenes and tumor-suppressor genes that are normally associated with cell transformation [(RAS, c-Myc, murine sarcoma 3611 oncogene (RAF), human epidermal growth factor receptor-2 (HER-2/neu), c-Jun, and steroid receptor coactivator (SRC)] regulate angiogenesis through upregulation of VEGF or downregulation of thrombospondin-1 (TSP-1), an angiogenesis suppressor [223,224]. Furthermore, mutated p53 upregulates VEGF and in contrast, wild-type p53 decreases VEGF production and increases TSP-1 [225]. Angiogenic factors such as VEGF, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) are released into the tumor microenvironment by

tumor or inflammatory cells in response to various stimuli, such as ROS [226]. The released growth factors activate endothelial cells that give rise to new blood vessels [227,228].

Monte et al. have demonstrated that lymphocyte-induced angiogenesis is triggered by ROS stimulation, and that this response can be blocked by the administration of a free radical scavenger to tumor bearing mice [229] [230]. In addition, the administration of H₂O₂ or an oxidative stress-producing drug (doxorubicin) to normal mice activated in vivo angiogenesis [229].

Due to reduced physiological tissue oxygen tension (hypoxia), which occurs during tumor initiation, tumors often become hypoxic. Under hypoxic conditions, cells activate signaling pathways, which regulate proliferation, angiogenesis, and death. Cancer cells have adapted to these pathways, effectively allowing tumors to survive and even grow under adverse hypoxic conditions [160]. This adaptation of tumor cells to hypoxia contributes to the malignant phenotype and to aggressive tumor progression [231], and low oxygen tension in tumors is associated with increased metastasis and poor survival of patients with several forms of squamous tumor [232,233]. HIF-1 α responds to these changes by specifically decreasing the oxygen (or hypoxia) level, and upregulating several genes to promote survival in low-oxygen conditions and thus promoting angiogenesis.

In conclusion, although previous sections indicate that all different sub-stages of tumor development are affected by ROS and inflammation, early stages of cancer development (e.g. cellular transformation), involving DNA damage, are however most affected by ROS generated inflammation. For example, colitis may develop into colon cancer after inflammatory infiltration, increased production of ROS, impairment of antioxidant defenses, DNA damage, and genetic and epigenetic alterations, resulting in the transformation of epithelial cells [234]. Or, bronchitis, which can lead to lung cancer, clearly links pro-oxidants, generated by cigarette smoke, to inflammation of the bronchus, and eventually transformation of lung cells into lung cancer [235]. Similarly pancreatitis and esophagitis, both induced by tobacco and alcohol, may transform normal tissue into pancreatic or esophageal cancer if the antioxidant system is not sufficiently effective [236,237].

4f. Chemoresistance

Despite many decades of research, the mechanisms underlying chemoresistance are still poorly understood. There is growing evidence that the inflammatory tumor microenvironment modulates not only cancer development but also cancer responsiveness and resistance to conventional anticancer therapies [238]. Experimental studies have led to the identification of various cancer cell-intrinsic resistance mechanisms, e.g., activation and/or overexpression of drug transporter proteins (e.g., P-glycoprotein), altered expression of detoxifying enzymes (e.g., glutathione S-transferase) or resistance to apoptosis/senescence pathways [239-242].

For example, an inflammatory response induces changes in expression and activity of multidrug-resistance (MDR)-associated protein transporters, greatly affecting drug responses [243,244]. It has been shown that acute inflammation suppresses the drug transporter P-glycoprotein (PGP) in the liver, whereas it activates PGP in kidneys, resulting in changes in the pharmacokinetics of the PGP substrate doxorubicin [245]. Likewise, expression of multidrug resistance-associated protein 1 (MRP1) is elevated in inflamed intestine of patients with Crohn's disease or ulcerative colitis [246]. Thus, enhanced states of inflammation influence proteins that are strongly linked with drug resistance.

In addition to the effects caused by inflammation, several chemotherapeutic agents have also been shown to activate the transcription factor NF- κ B in human lung and cervical cancers

and in T cells [247-249]. These agents are paclitaxel, vinblastine, vincristine, doxorubicin, daunomycin, 5-fluorouracil, cisplatin, and tamoxifen. Activation of NF- κ B by these agents has been linked in turn with chemoresistance through serine phosphorylation of inhibitor of κ B α (I κ B α) [250,251]. Various in vitro studies have supported a link between NF- κ B activation, cytokine production and chemoresistance. One pathway via which NF- κ B can be activated is the Toll-like receptor (TLR) pathway. TLRs generally signal via the adapter protein myeloid differentiation primary response gene 88 (MyD88) leading to activation of NF- κ B and production of pro-inflammatory cytokines. Activation of TLR signaling in ovarian cancer cell lines by exogenously added LPS resulted in an activated NF- κ B pathway, which promoted secretion of proinflammatory cytokines and subsequently conferred resistance to paclitaxel [252,253]. Also, TNF receptor signaling promotes NF- κ B activation and has been linked with chemoresistance. For example, exposure of breast cancer cells to exogenously added TNF α results in selection for breast cancer cells that overexpress NF- κ B, leading to increased cancer cell survival and resistance to ionizing radiation [254]. At the same time, cytokines produced by stromal cells in the tumor microenvironment (e.g., IL-1 or TNF α) could potentially activate the NF- κ B pathway in cancer cells and thus contribute to chemoresistance. These data call for functional in vivo studies to elucidate the involvement of the inflammatory tumor microenvironment in NF- κ B-dependent chemoresistance.

Another mechanism that might be involved in chemoresistance is increased levels of GSH in cancer cells [92]. In particular, the overexpression of glutathione S-transferases (GST), the enzymes that catalyse the conjugation of reduced glutathione to electrophilic [255], as well as efflux pumps, may reduce the reactivity of various anticancer drugs [256]. The increase of the GST levels occurs by transcriptional activation mediated by the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) [257]. Indeed, using genetic manipulation, Lau et al. have demonstrated a strong positive correlation between Nrf2 levels and resistance of three cancer cell lines to chemotherapeutic drugs such as cisplatin, doxorubicin, and etoposide [258]. Chemical activation of Nrf2 by pretreatment with tertiary-butylhydroquinone (tBHQ) also increased survival of neuroblastoma cells in response to the three drugs tested [259]. Consistent with these findings, the role of Nrf2 in determining efficacy of cisplatin was also demonstrated in ovarian cancer cells using siRNA knockdown of Nrf2 [260]. Moreover, many kelch-like ECH-associated protein 1 (Keap1) mutations or loss of heterozygosity in the Keap1 locus have been identified in lung cancer cell lines or cancer tissues [261,262]. Keap1 mutations or loss of heterozygosity resulted in inactivation of Keap1 or a reduced expression of Keap1, which upregulated the protein level of Nrf2 and transactivation of its downstream genes [261,262]. Similar to Nrf2, the protective effect of heme oxygenase-1 (HMOX-1, or HO-1) in normal cells may protect from oxidative stress-related diseases. However, such an effect is undesirable in cancer because it provides a selective advantage for cancer cells to survive. Consistent with this notion, HMOX-1 has been found to be overexpressed in various tumor types. It is believed that overexpression of HMOX-1 facilitates cancer cell growth and survival in many ways, such as stimulating rapid growth of cancer cells, enhancing cancer cell resistance to stress and apoptosis, promoting angiogenesis of tumors, and aiding in metastasis of tumors [263]. In addition to HMOX-1, other Nrf2-downstream genes such as Prx1, GPx, and thioredoxin reductase (TrxR) were also upregulated in many cancer cells or tissues and may contribute to chemoresistance [264-266]. In ovarian cancer, constitutive activation of ERK activity has been associated with high tumorigenicity and chemoresistance [267,268]. In addition, functional analyses employing knockdown of MKP3, a member of the subfamily of protein tyrosine phosphatases known as dual-specificity phosphatases (MKPs) [269,270], and ectopic overexpression revealed the role of MKP3 in negatively regulating ERK1/2 activity and inhibiting tumorigenicity and chemoresistance in vitro and in vivo. MKP3 is capable of

dephosphorylating ERK1/2 by protein-protein interactions via mitogen-activated protein kinase interaction motif within the N-terminal ERK1/2-binding domain [271].

4g. Radioresistance

Acquired tumor radioresistance can be induced during radiotherapy owing to tumor repopulation [272]. Although tumor radioresistance stands as a fundamental barrier limiting the effectiveness of radiation therapy, the exact molecular mechanisms underlying the radioadaptive response are largely unknown (Figure 4). Olivieri et al. [273] first described an adaptive response of human lymphocytes to ionizing radiation. Since then, a substantial number of reports have made a strong case for the existence of cellular radioprotective mechanisms that can be activated in response to a small dose of ionizing radiation. It is assumed that a specific pro-survival signaling network is induced in irradiated mammalian cells.

The elevated basal NF- κ B activity in certain cancers has been linked with tumor resistance to chemotherapy and radiation [274]. NF- κ B in adaptive radioresistance is evidenced in mouse epidermal cells [275] and human keratinocytes, and inhibition of NF- κ B blocks the adaptive radioresistance [275]. Human breast cancer cells treated with fractional γ -irradiation show an enhanced clonogenic survival and NF- κ B activation [276,277]. Blocking NF- κ B inhibited the adaptive radioresistance. These results provide the first evidence that activation of NF- κ B is required for signaling the radio-adaptive resistance by exposure to radiation. Together with the assumption that NF- κ B is able to regulate more than 150 effector genes, these results suggest that NF- κ B plays a key role in tumor radioadaptive resistance under fractional ionizing radiation. Furthermore, in a study [278] that immunocytochemically examined the levels of activated NF- κ B protein in pretreatment cancer specimens and in resected specimens of patients with chemoradiotherapy resistance, the cancers expressed higher levels of cytoplasmic NF- κ B than did the adjacent nonmalignant mucosa. Furthermore, Sandur et al. suggest that transient inducible NF- κ B activation provides a prosurvival response to radiation that may account for the development of radioresistance [279].

On the other hand, hypoxia is a principal signature of the tumor microenvironment and is considered to be the most important cause of clinical radioresistance and local treatment failure. The response of cells to ionizing radiation is strongly dependent upon oxygen, which is traditionally explained by the “oxygen fixation hypothesis” [280]. Oxygen is so far the best radiosensitizer. De Ridder et al. demonstrated that iNOS, activated by pro-inflammatory cytokines, can radiosensitize tumor cells through endogenous production of NO [280]. They further observed that this radiosensitizing effect is transcriptionally controlled by hypoxia and by NF- κ B. Consistently, NF- κ B inhibition has been used as an approach to radiosensitize tumor cells, aiming at stimulating apoptosis and inhibiting DNA repair. Moreover, the inflammatory mediators TNF α and NO have been repeatedly used as targets to radiosensitize tumor cells [281-285].

4h. Stem cell survival

Cancer stem cells (CSCs) are cancer cells that have the ability to generate tumors through the processes of self-renewal and differentiation into multiple cells. Such cells persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. The existence of CSCs may have several implications in cancer treatment, including disease identification, selection of drug targets, prevention of metastasis, and development of new intervention strategies.

The first conclusive evidence for CSCs was published in 1997 [286], and to date CSCs have been isolated from both leukemias and a variety of solid tumors, including breast, brain, pancreatic, prostate, ovary, and colon cancers [287-293]. The pathways that regulate self-renewal of CSCs include wnt (Wnt), Notch, Hedgehog, and tumor-suppressor genes such as PTEN and TP53 (tumor protein 53) [294]. Although redox balance plays an important role in the maintenance of stem cell self-renewal and in differentiation, redox status in CSCs has yet to be explored. However, given the similarity between normal stem cells and CSCs and the fact that redox status plays an important role in cancer cell development, it is tempting to speculate that redox status may have a role in CSC survival. A recent study by Diehn et al. demonstrated that, similar to normal stem cells, subsets of CSCs in human and murine breast tumors have lower ROS levels than do the corresponding non-tumorigenic cells [295]. The group further showed that lower levels of ROS were associated with increased free radical scavenging systems and that pharmacologic depletion of these scavengers significantly decreased clonogenicity and resulted in radiosensitization of CSCs. Additionally, two studies showed that CD133+ CSCs conferred chemoresistance to cisplatin and doxorubicin (known ROS generators) in ovarian cancer cells [296] and hepatocellular carcinoma [297], respectively. These studies further indicate that redox status may be important in maintaining CSC survival.

4i. Stromal cell signaling

Cancer progression must involve both genetic and behavioral changes in cancer cells, and these changes are in part driven by the cancer-associated stromal cells and tumor microenvironment [298,299]. The stromal component of the normal prostate epithelium, for example, consists of smooth muscle, fibroblasts, vascular endothelial cells, nerve cells, inflammatory cells, insoluble matrix, and soluble factors [300]. Studies by De Marzo et al. highlight the role of inflammation in prostate cancer, suggesting that atrophic lesions are an early event in prostate carcinogenesis [301]. The macrophages in the tumor microenvironment produce ROS and RNS. The resulting increases in superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and free iron damage DNA, causing genetic mutations and initiating cancer progression. Tissue and cell recombination studies demonstrate the important regulatory role of fibromuscular stroma and stromal fibroblasts in prostate development and prostate carcinogenesis [300]. Cancer cells and stromal cells interact through physical contact or through soluble factors or insoluble extracellular matrix (ECM) factors. These stromal fibroblasts, which interact with cancer cells, have increased levels of brain-derived neurotrophic factor, chemokines, CC chemokine ligand 5 (CCL5) and CXC chemokine ligand 5 (CXCL5), versican, tenascin, connective tissue growth factor, stromal cell derived factor-1/ CXC chemokine ligand 12 (SDF-1/CXCL12), and HIF-1 α [302]. Other studies have demonstrated the role of stromal soluble factors interacting with receptors on prostate cancer cells. The stromal factors include VEGF, bFGF, hepatocyte growth factor/ scatter factor (HGF/SF), transforming growth factor- β (TGF- β), insulin like growth factor-1 (IGF-1), IL-6, and keratinocyte growth factor (KGF) [303].

Several studies have found that tumors promote a constant influx of myelomonocytic cells that express inflammatory mediators supporting pro-tumoral functions. Myelomonocytic cells are key orchestrators of cancer-related inflammation associated with proliferation and survival of malignant cells, subversion of adaptive immune response, angiogenesis, stroma remodeling, and metastasis formation [304].

Tumor-derived factors, which cause sustained myelopoiesis, accumulation, and functional differentiation of myelomonocytic cells, provide an essential support for the angiogenesis and the stroma remodeling required for tumor growth [305,306]. In addition, it has long been known that tumor growth is promoted by tumor-associated macrophages (TAM), a major leukocyte population present in tumors [65,307-310]. Accordingly, in many but not

all human tumors, a high frequency of infiltrating TAM is associated with poor prognosis. A model by which macrophages promote tumor invasion and metastasis includes expression of their proteolytic activity and subsequent breakdown of the basement membrane around the preinvasive tumors, thereby enhancing the ability of tumor cells to escape into the surrounding stroma [311]. In lung cancer, for example, TAM may favor tumor progression by contributing to stroma formation and angiogenesis through their release of platelet-derived growth factor, in conjunction with TGF- β production by cancer cells [310]. TAM produce several MMPs, such as MMP-2 and MMP-9, that degrade proteins in the extracellular matrix and also produce activators of MMPs, such as chemokines.

5. Conclusion

This review clearly implicates the role of ROS in different phases of tumorigenesis. Therefore, targeting redox-sensitive pathways and transcription factors offers great promise for cancer prevention and therapy. Numerous agents have been identified that can interfere with redox cell signaling pathways [9,312,313]. These include nutraceuticals derived from fruits, vegetables, spices, grains, and cereals. They have been shown to suppress tumorigenesis in preclinical models. Whether these agents can inhibit tumor growth in patients remains to be elucidated.

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6. Abbreviations

Akt	AKT8 virus oncogene cellular homolog
AP-1	activator protein-1

APC	adenomatous polyposis coli
ATF-2	activating transcription factor-2
Bad	Bcl-XL/Bcl-2-associated death promoter
BH3	Bcl-2 homology3
BRCA1	breast cancer susceptibility gene 1
CDKN-2	cyclin-dependent kinase inhibitor-2
COX-2	cyclooxygenase-2
CCL5	CC chemokine ligand 5
CSCs	cancer stem cells
Cu-ZnSOD	copper-zinc superoxide dismutase
CXCL5	CXC chemokine ligand 5
CXCR4	CXC chemokine receptor 4
ECM	extracellular matrix
EC-SOD	extracellular-superoxide dismutase
eNOS	endothelial nitric oxide synthase
ERK/MAPK	extracellular signal-regulated kinase/ mitogen-activated protein kinase
FGF	fibroblast growth factor
HIF-1α	hypoxia inducible factor-1 α
Flk1/KDR	fetal liver kinase 1/ kinase insert domain receptor
GPx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulphide
GTPase Rac1	guanosine triphosphatase Rac1
HER-2	human epidermal growth factor receptor-2
HGF/SF	hepatocyte growth factor/ scatter factor
HIF-1α	hypoxia-inducible factor-1 α
hMLH1	human mutL homolog 1
HMOX-1	heme oxygenase-1
4-HNE	4-hydroxynonenal
H₂O₂	hydrogen peroxide
Hsp27	heat shock protein27
ICAM-1	intercellular adhesion molecule-1
IGF-1	Insulin like growth factor-1
IκBα	inhibitor of κ B α
IL-1	interleukin-1
IL-6	interleukin-6

IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
IFN	interferon
JNK	c-Jun N-terminal kinase
c-JUN	cellular Ju-nanna
KGF	keratinocyte growth factor
Keap1	Kelch-like ECH-associated protein 1
LPS	lipopolysaccharide
MDR	multidrug-resistance
MDM2	murine double minute 2
MKPs	mitogen-activated protein kinase phosphatases
MMPs	metalloproteinases
Mn-SOD	manganese-superoxide dismutase
MRP1	multidrug resistance-associated protein 1
Myc	avian myeloblastosis virus oncogene
MyD88	myeloid differentiation primary response gene 88
NAC	N-acetylcysteine
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κ B
NO	nitric oxide
Nox	NADPH oxidase
Nrf2	NF-E2 related factor-2
8-OHdG	8-hydroxydeoxyguanosine
p27Kip1	p27 kinase inhibitor protein
PDGF	platelet-derived growth factor
PGP	P-glycoprotein
PI3K	phosphoinositide 3- kinase
PKB/Akt	protein kinase B/AKT8 virus oncogene cellular homolog
PMA	phorbol 12-myristate 13- acetate
PPAR-γ	peroxisome proliferator-activated receptor-γ
PTEN	phosphatase and tensin homolog deleted from chromosome 10
Prx	peroxiredoxins
RAS	rat sarcoma viral oncogene
RAF	murine sarcoma 3611 oncogene
Rb	retinoblastoma protein

ROS	reactive oxygen species
RNS	reactive nitrogen species
SDF-1/CXCL12	stromal cell derived factor-1/ CXC chemokine ligand 12
SOD	superoxide dismutase
SRC	steroid receptor coactivator
STAT3	signal transducer and activator of transcription 3
TAM	tumor-associated macrophages
tBHQ	tertiary-butylhydroquinone
TGF-β	transforming growth factor- β
TLR	toll-like receptor
TNF	tumor necrosis factor
TSP-1	thrombospondin-1
TrxR	thioredoxin reductase
VEGF-A	vascular endothelial growth factor-A
Wnt	wint

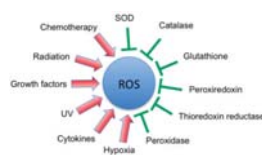


Figure 1. Schematic representation of various activators and inhibitors of reactive oxygen species production

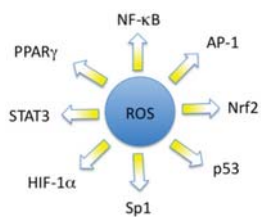


Figure 2. Schematic representation of various transcription factors that are modulated by reactive oxygen species

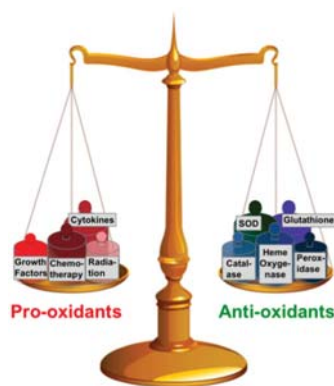


Figure 3. Model of a balance between pro-oxidants and anti-oxidants

Under normal conditions, anti-oxidants outbalance pro-oxidants, but under oxidative conditions, pro-oxidants prevail over anti-oxidants, which can lead to many inflammatory diseases including cancer.

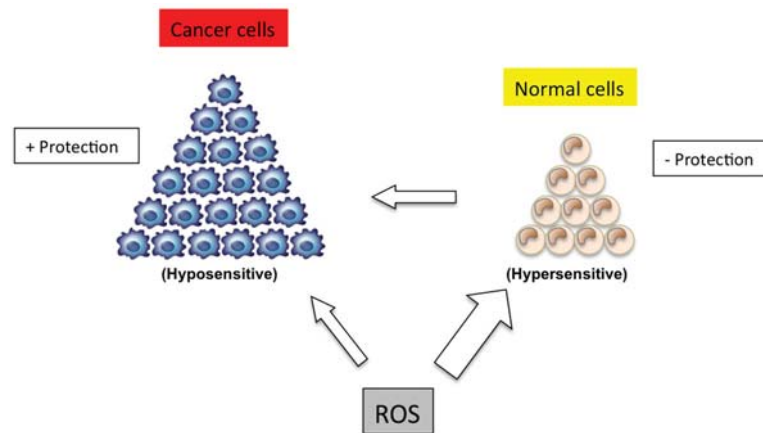


Figure 4. Model of the sensitivity of normal cells versus cancer cells to reactive oxygen species
Normal cells are hypersensitive to ROS if not adequately protected by anti-oxidant mechanisms, which may lead to cancer formation. Cancer cells, on the other hand, have upregulated antioxidant mechanisms (glutathione, SOD, catalase, and others) that will protect them against ROS, as can be observed in, for example, the case of radioresistance.

Table 1
A partial list of diseases that have been linked to reactive oxygen species

Disease	Reference
Acute Respiratory Distress Syndrome	[16]
Aging	[17]
Alzheimer	[18,19]
Atherosclerosis	[20]
Cancer	[21-23]
Cardiovascular Disease	[24,25]
Diabetes	[26]
Inflammation	[27]
Inflammatory Joint Disease	[28]
Neurological Disease	[29]
Obesity	[30,31]
Parkinson	[32,33]
Pulmonary fibrosis	[34,35]
Rheumatoid arthritis	[36]
Vascular Disease	[37,38]

Table 2
A partial list of cancers that have been linked to reactive oxygen species

Cancer	Reference
Bladder Cancer	[39]
Brain Tumor	[40]
Breast Cancer	[41]
Cervical Cancer	[42]
Gastric (Stomach) Cancer	[43]
Liver Cancer	[44]
Lung Cancer	[45]
Melanoma	[46]
Multiple Myeloma	[47]
Leukemia	[48]
Lymphoma	[49]
Oral Cancer	[50]
Ovarian Cancer	[51]
Pancreatic Cancer	[52]
Prostate Cancer	[10]
Sarcoma	[53]

Table 3
A partial list of signaling pathways linked to reactive oxygen species

Signaling intermediate	Reference
AHR	[98]
AP-1	[99,100]
ATM	[101]
cAMP	[102]
cAMP-dependent PKA	[103]
CDK5	[104]
Chemokine	[70]
c-myc	[99]
CREB	[103]
Cyclins and Cell Cycle Regulation	[105]
Cytokine Network	[66]
DNA Methylation	[106]
DNA Repair Mechanism	[107]
EGF	[108]
eNOS	[109]
ERK	[110]
Fas	[111]
FOXO	[112]
HIF-1 α	[113]
HO-1	[114]
IL-10	[115]
iNOS	[109]
Integrin	[116]
Interferon	[117]
JAK/STAT	[118]
JNK	[119]
MAPK	[110]
Mismatch Repair	[120]
mTor	[121]
NAD(P)H quinone oxidoreductase 1	[122]
NF- κ B	[123]
Nfr2	[124]
PI3K/Akt	[125]
p38	[126]
p53	[127,128]
PKC	[129]
PPAR γ	[130]
PTEN	[131]
PTPs/PTKs	[132]

Signaling intermediate	Reference
Sp1	[133]
TNF	[5]
VEGF	[134]
WNT	[135,136]

Exhibit 104

The Role of the Mediators of Inflammation in Cancer Development

José Veríssimo Fernandes · Ricardo Ney Oliveira Cobucci ·
Carlos André Nunes Jatobá · Thales Allyrio Araújo de Medeiros Fernandes ·
Judson Welber Veríssimo de Azevedo · Josélio Maria Galvão de Araújo

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Abstract Epigenetic disorders such as point mutations in cellular tumor suppressor genes, DNA methylation and post-translational modifications are needed to transformation of normal cells into cancer cells. These events result in alterations in critical pathways responsible for maintaining the normal cellular homeostasis, triggering to an inflammatory response which can lead the development of cancer. The inflammatory response is a universal defense mechanism activated in response to an injury tissue, of any nature, that involves both innate and adaptive immune responses, through the collective action of a variety of soluble mediators. Many inflammatory signaling pathways are activated in several types of cancer, linking chronic inflammation to tumorigenesis process. Thus, Inflammatory responses play decisive roles at different stages of tumor development, including initiation,

promotion, growth, invasion, and metastasis, affecting also the immune surveillance. Immune cells that infiltrate tumors engage in an extensive and dynamic crosstalk with cancer cells, and some of the molecular events that mediate this dialog have been revealed. A range of inflammation mediators, including cytokines, chemokines, free radicals, prostaglandins, growth and transcription factors, microRNAs, and enzymes as, cyclooxygenase and matrix metalloproteinase, collectively acts to create a favorable microenvironment for the development of tumors. In this review are presented the main mediators of the inflammatory response and discussed the likely mechanisms through which, they interact with each other to create a condition favorable to development of cancer.

Keywords Inflammation and cancer · Inflammation mediators · Mechanisms of tumorigenesis

J. V. Fernandes · J. M. G. de Araújo
Post-Graduate Program in Biological Sciences, Federal University of Rio Grande do Norte, Natal, RN, Brazil

J. V. Fernandes (✉) · J. M. G. de Araújo
Department of Microbiology and Parasitology, Federal University of Rio Grande do Norte, Campus Universitário, 59072-910 Natal, RN, Brazil
e-mail: joseverissimo1951@gmail.com

R. N. O. Cobucci
Maternidade Escola Januário Cicco, Federal University of Rio Grande do Norte, Natal, RN, Brazil

C. A. N. Jatobá
Department of Pathology, Federal University of Rio Grande do Norte, Natal, RN, Brazil

T. A. A. de Medeiros Fernandes
Department of Biomedical Sciences, University of Rio Grande do Norte State, Mossoró, RN, Brazil

J. W. V. de Azevedo
Hospital Universitário, Federal University of Triângulo Mineiro, Mina Gerais, Brazil

Abbreviations

AA	Arachidonic acid
AP-1	Activator protein 1
APC	Antigen-presenting cell
cAMP	Cyclic AMP
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cHL	Classical Hodgkin lymphoma
CLRs	C-type lectin receptors
COX	Cyclooxygenase
CRC	Colorectal cancer
CXC	Chemokine receptors
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FOXP3	Forkhead box P3

GPCRs	G protein-coupled
HPV	Human papillomavirus
ICC	Invasive cervical cancer
IFN	Interferon
IL	Interleukin
MHC	Major histocompatibility complex
miRNAs	MicroRNAs
MM	Multiple myeloma
MMPs	Enzymes matrix metalloproteinase matrix
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NLRs	NOD-like receptors
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
p53	Tumor protein p53
PAMPs	Pathogen-associated molecular patterns
PGs	Prostaglandins
PRRs	Pattern recognition receptors
PTGER	Prostaglandin receptor
PTGES	Terminal prostaglandin synthase enzyme
PubMed	US National Library of Medicine
RLRs	RIG-like receptors
ROS	Reactive oxygen species
STAT	Signal transducers and activators of transcription
TCR	T Cell Receptor
TGF	Transforming growth factor
Th cells	T helper cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
Txs	Thromboxanes

Introduction

The primary functions of inflammation are rapidly destroying or isolating the underlying source of the disturbance and then restoring homeostasis so that, being regulated properly, behaves as an adaptive mechanism. One indication of this is the fact that humans with primary genetic defects in the components of inflammation have increased risk of serious infections. A similar phenomenon was observed in animals with defects in genes encoding pro-inflammatory cytokines [1]. Moreover, immunologically relevant genes whose dysfunction leads to spontaneous inflammation are not expressed under normal conditions, suggesting that the inflammatory response is suppressed to maintain health since its deregulation can have devastating effects for the host, resulting in collateral damage and pathology [2]. Thus, despite being a designed response to eliminate pathogens and other agents harmful to the host, the inflammation when deregulated or

inappropriately maintained has the potential to cause injury, necrosis, and malignant transformation [3].

Much evidence supports the hypothesis that inflammation participates in providing conditions that lead to cancer. An unresolved inflammation due to any failure in precise control of the immune response can lead to alterations in expression of cancer-related genes and posttranslational modification in cellular proteins involved in the cell cycle, DNA repair, and apoptosis favoring the development of cancer [4]. Currently, it is well established that chronic inflammation is strongly associated with several human cancers, since it leads to the release of pro-inflammatory cytokines, and other immunomodulatory, creating a favorable microenvironment for tumor progression and metastasis [5].

The inflammation generates oxidative stress, which in turn increases inflammation, so that the two are common denominators in carcinogenesis. Oxidative stress generates reactive oxygen species (ROS) that causes DNA damage and activates signaling pathways that deregulate the cell cycle and hence increase the risk of development of cancers. There is a cross-talk between these two mediators, where ROS and inflammation potentiate each other to ultimately cause cancer [6]. Thus, the inflammatory response plays key roles at different stages of tumor development, besides affecting immune surveillance. Immune cells that infiltrate into tumors establish a cross-talk with cancer cells to orchestrate interactions between different mechanisms, which together can lead to the formation of tumors. This review presents a discussion of some mediators of inflammation and the molecular events through which communication is established between immune and tumor cells, as key mechanisms regulating the effects of inflammation and immunity on tumor development.

The literature review was conducted in the electronic databases PubMed (National Institutes of Health), Scopus (Elsevier), and Web of Knowledge (Thomson Reuters), using the following keywords: carcinogenesis, Inflammation and cancer. The databases retrieved hundreds of articles, and we selected those that we thought to be most relevant to our purpose.

Mediators Involved in the Inflammation and Carcinogenesis

The Infections and chronic inflammation contribute to about 1 in 4 of all cancer cases. Mediators of the inflammatory response, such as: cytokines, chemokines, free radicals, prostaglandins, growth factors and enzymes as cyclooxygenase (COX) and matrix metalloproteinase, can induce genetic and epigenetic changes, that result in alterations in critical pathways responsible for maintaining the normal cellular homeostasis and can leading to the development and progression to cancer [7–9].

Cytokines and chemokines are involved in many aspects of growth, differentiation and cell activation. Table 1 summarizes the actions of the main cytokines that play some role in the activation or regulation of the inflammatory response and that contribute in some way to the process of tumorigenesis.

Chemokines are key players of the cancer-related inflammation, whereas their respective receptors and ligands are the downstream genetic events that cause neoplastic transformation and which are abundantly expressed in chronic inflammation, increasing susceptibility to cancer. The components of the chemokine system affect different routes of tumor progression, including leukocyte recruitment, neo-angiogenesis, proliferation, survival, invasion, and metastasis of tumor cells. Preclinical and clinical trials indicate that the intervention in the chemokine system can be a valuable tool for the development of future therapeutic strategies against cancer [35].

It has been shown that the CXCR2 chemokine receptor and its ligands promote angiogenesis and leukocyte infiltration in the tumor microenvironment. In the acidic and hypoxic conditions of the tumor microenvironment, up-regulating the expression of CXCR4 creates a gradient prepared by CXCL12 for migration of tumor-associated fibroblasts (CAF). The axis CXCL12-CXCR4 facilitates metastasis to distant organs and the CCL21-CCR7 chemokine ligand-receptor pair favors metastasis to lymph nodes. These two chemokine ligand-receptor systems are common key mediators of tumor cell metastasis for several malignancies [36].

It has been shown that cancer cells secrete, or induce fibroblasts to secrete the chemokine CCL5, which acts in an autocrine or paracrine manner on tumor cells, which express their receptor (CCR5). This promotes the proliferation of these cells and recruitment of T-reg cells and monocytes to induce activation of osteoclasts and bone metastases, by inducing neoangiogenesis, and to facilitate the spread of tumor cells for distant organs. It is believed that CCL5, produced by cells of classical Hodgkin lymphoma (cHL), may represent an autocrine growth factor of the tumor cells by creating a microenvironment conducive to tumor progression, whereas CCL5 secreted by T cells or fibroblasts may represent a paracrine growth factor. TCD4⁺ cells expressing CD40L increase the secretion of CCL5 by cHL cells and induce secreting CCL5 by fibroblasts, which promote the recruitment of activated fibroblasts by cHL cells, which in turn recruit T-reg cells, eosinophils, and mast cells [35].

It has been observed that CXCL8, a chemokine of the CXC family, exerts its effects through signaling two G-coupled receptors, CXCR1 and CXCR2 protein. Elevated CXCL8 signaling - CXCR1 / 2 within the tumor microenvironment of various types of human cancers promotes tumor progression through the activation of signaling pathways involved in activation of proliferation, survival, angiogenesis, migration, and cell invasion, through transactivation of the epidermal growth factor receptor (EGFR) [5].

The Role of Transcription Factors NF- κ B

The NF- κ B family of transcription factors has been recognized as a crucial player in many steps of cancer including initiation and progression, cooperating with multiple other signaling molecules and pathways. This action is mediated by other transcription factors such as STAT3 and p53 or the ETS-related gene ERG, which directly interacts with NF- κ B subunits or affects NF- κ B target genes. Crosstalk can also occur through different kinases, such as GSK3- β , p38, or PI3K, which modulate NF- κ B transcriptional activity or affect upstream signaling pathways. Other classes of molecules that can also act in the integration of these mechanisms involving NF- κ B are reactive oxygen species and miRNAs [37].

It is well known that NF- κ B regulate the expression of numerous cytokines and adhesion molecules which are critical elements involved in the regulation of immune responses [38]. Furthermore, it coordinates the central signaling pathways of activation of the innate and adaptive immune responses, and that STAT3 regulates the expression of various genes in response to cellular stimuli, playing a key role in cell growth and apoptosis. It has been shown that STAT3 is constitutively activated in many human cancers, including gastric cancer and plays crucial roles in modulating proliferation and survival, cancer cells as well as creating a favorable microenvironment to the formation of metastasis [39].

The activation and interaction between STAT3 and NF- κ B have been widely investigated in human cancers such as colon, stomach, and liver cancers. It has been shown that the interaction between these two transcription factors play a vital role in controlling the communication between inflammatory cells and cancerous cells. NF- κ B and STAT3 are the main two factors that control the capacity of pre-neoplastic and malignant tumor cells to resist immune surveillance by regulating apoptosis, angiogenesis, and tumor invasion. The understanding of the molecular mechanisms of NF- κ B and STAT3 cooperation in cancer development will provide opportunities for the design of new chemo-preventive and chemotherapeutic approaches [40].

The Role of Matrix Metalloproteinase and Cyclooxygenases in the Carcinogenesis

The matrix metalloproteinases (MMPs) are members of the metzincin group of proteases, and constitute a family of zinc-dependent proteolytic enzymes that degrade various components of the extracellular matrix (ECM). Due to their broad spectrum of substrate specificity, MMPs contribute to the homeostasis of many tissues and participate in diverse physiological processes, such as bone remodeling, angiogenesis, wound healing, and immunity. However, the unregulated

Table 1 The role of some cytokines in cancer

Cytokine	Role in cancer development	Ref.
Interleukin-1 β (IL-1 β)	Suppression of p53 expression; Cancerous epithelial cells uses IL-1 β as a communication factor instructing stromal fibroblasts, whose expression of p53 was suppressed, creating an inflammatory microenvironment and protumorigenic	[10]
Tumor necrosis factor- α (TNF- α)	Creation of a tumor microenvironment that stimulates the growth and survival of tumor cells through the induction of gene encoding NF- κ B dependent antiapoptotic molecules. Furthermore, It cause inflammatory cell infiltration in tumors and promotes angiogenesis, invasion and migration of tumor cell, and suppress cytotoxic T lymphocytes and activated macrophages. TNF- α also contributes to the initiation of tumors through the stimulation of production of genotoxic molecules such as nitric oxide (NO) and ROS, which may cause DNA mutations.	[11–13]
Transforming growth factor- β (TGF- β)	TGF- β is essentially an inhibitory cytokine with an anti-inflammatory and immunosuppressive action, and has a central role in the proliferation and function of Treg cells. Changes in its signaling pathways are often observed in human cancer. These alterations attenuate the TGF- β tumor suppressive effects, promoting tumor progression and metastasis. The carcinoma often secrete this cytokine in excess, resulting in increased epithelial-mesenchymal transition with tissue invasion and metastasis.	[14–16]
Interleukin-6 (IL-6)	Stimulation of angiogenesis, promotion of cell proliferation and increased survival of malignant cells, besides inhibit the apoptosis of cancer cells. Clinical studies have shown that high serum levels of IL-6 are associated with advanced stages of various cancers.	[17, 18]
Interleukin-10 (IL-10)	Inhibition of IFN- γ production by Th1 cells as well as production of inflammatory cytokine, including TNF- α , IL-6, and IL-12. Therefore, it is involved in the inhibition of tumor development and progression. However, depending on the context in witch it acts, this cytokine can have action against or favorable to development of tumor. Its presence in the inflammatory microenvironment of the tumor can eliminate the anticancer action of the Th1 response. On the other hand, IL-10 and Tregs also suppress the activity of Th17, which is associated with poor prognosis in several types of cancer.	[19–25]
Interleukin-17 (IL-17)	Induction of many proinflammatory mediators, including TNF- α , IL-1 β , and IL-6, suggesting a role in locating and amplifying the inflammation. Besides, several studies have shown large amounts of Th17 cells infiltrated in tumors and high levels of expression of IL-17 in the serum of patients with several types of tumors, suggesting an important role in the tumorigenesis. The Th17/Treg balance was also broken in the peripheral blood of cervical cancer patients.	[26–28]
Interleukin-12 (IL-12)	IL-12 has a protective activity against cancer, acting to prevent initiation, growth, and metastasis of tumors. It stimulates the cytotoxic activity and production of IFN- γ and TNF- α from NK and TCD8 cells, promoting a TH1 immune response, besides an antiangiogenic function. Recently, it has become evident the balance between IL-12 and IL-23 (a promoter of Th17 immune response) is important in the carcinogenesis process.	[29, 30]

Table 1 (continued)

Cytokine	Role in cancer development	Ref.
Interleukin-18 (IL-18)	IL-18 acts in synergy with IL-12 to induces Th1 immune response against cancer. The systemic administration of IL-18 has been shown to have significant antitumor activity in several preclinical animal models. However, its expression and secretion has been observed in several types of immune cells promoting cancer. Its levels has also been elevated in patients with squamous cell carcinoma of the skin.	[31–34]

activity of MMPs leads to pathological conditions such as arthritis, inflammation, and cancer [41, 42].

They are key regulators of ECM and basement membranes, contributing to the development and progression of human malignant tumors due to their interaction with the receptors for growth factors, cytokines, chemokines, cell adhesion molecules, apoptotic ligands, and angiogenic factors [43, 44].

There are several different types of MMPs, including MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, and MT1-MMP, which are stimulated and activated by various mechanisms in vascular tissues. Once activated, MMPs degrade ECM proteins and other related signaling molecules, promoting abnormal angiogenesis and remodeling of vascular tissue, and facilitating recruitment of stem / progenitor cells, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs). The changes in the behavior of these cells contribute to the pathogenesis of various disorders [43].

MMPs regulate inflammation by substrate processing of a range of novel substrates including chemokines, growth factors, receptors, binding proteins, proteases, protease inhibitors, and extra- and intracellular multifunctional proteins [45]. MMP-1 and MMP-13 are collagenases that degrade ECM, especially the collagens of type I, II, and III, which are the main components of the interstitial stroma. In colorectal cancer (CRC), the expression of MMP-1 is correlated with a more advanced stage of disease and with poor prognosis. It has been observed that the level of invasion of the lymph nodes by metastasis in CRC were associated with elevated levels of MMP-1. It has been shown that the expression of MMP-13 may be related to tumor biological aggressiveness and used to aid in predicting patient's poor prognosis. In fact, the expression of MMP-13 was correlated with the decreased survival of patients with CRC [46].

MMP-2 and MMP-9 are gelatinases whose main substrate is type IV collagen and gelatin, but they also have proteolytic activity against other extracellular matrix molecules. Higher levels of expression of these enzymes were found in the plasma of patients with CRC that have metastasis in lymph nodes compared with those without lymph node metastases. MMP-7 is a matrilysin whose expression has been observed in about

80 % of all cases of CRC, and its serum levels are associated with the progression of CRC and decreased survival rate. MMP-7 promotes cancer invasion through cleavage of ECM proteins and activates other MMPs, including proMMP-2 and proMMP-9, to promote invasion of cancer cells. MMP-12 is a metalloelastase expressed predominantly in the macrophage, and it is able to degrade many different substrates and seems to have a protective function in CRC, since its inhibition was considered potentially harmful to the patient with this pathology [44].

On the other hand, the cyclooxygenases are enzymes that convert free arachidonic acid (AA) into prostanoids, including prostaglandins (PGs) and thromboxanes (Tx). There are two isoforms of COX designated, COX-1 and COX-2, being COX-2 the most strongly linked to development and progression of cancer [47, 48]. High expression levels of COX-2 are found in the tissue of colorectal cancer (CRC) and are associated with less survival of patients with CRC [49]. The clinical and epidemiological studies and animal experiments indicate that non-steroidal anti-inflammatory drugs (NSAIDs) are among the most promising chemopreventive agents for this disease. The NSAIDs exert their anti-inflammatory and anti-tumor effects mainly by inhibiting the action of COX-2, leading to reduced production of prostaglandins [50].

In cells of invasive cervical cancer (ICC), E5, E6, and E7 HPV 16 oncogenes were able to induce the COX/prostaglandin inflammatory axis by increasing the expression of the COX-2 gene [9]. This suggests a direct link between HPV oncogene and activation of an inflammatory response, a potent factor in promoting cancer. Thus, although the initial HPV infection is not associated with inflammation, it is believed that, after integration of the virus into the cell genome, viral persistence occurs, followed by malignant transformation of the infected cell. This occurs due to the activation of inflammatory pathways such as COX-prostaglandin promoting an infiltration of inflammatory and immune cells, creating a favorable microenvironment for tumor progression [51].

Both COX 1 and 2 are significantly represented in cells of ICC, and the products of HPV oncogene and of the PGE2 gene can regulate the expression of the prostaglandin receptor (PTGER) [52]. Furthermore, it was demonstrated that E5 of

the HPV16 protein regulates the expression of PTGER4 in cells of ICC in a way that is dependent on PGE2 production of cyclic AMP (cAMP). This suggests that increased levels of PGE2 on ICC may regulate the function of neoplastic cells in an autocrine or paracrine manner, through the expression of high levels of PTGER2 and PTGER4 prostaglandin receptors [53].

The Role of microRNAs in the Carcinogenesis

MicroRNAs (miRNAs) are small noncoding single-stranded RNAs, which are highly conserved during evolution, and controls the gene expression by degrading the corresponding mRNA, destabilizing and/or inhibition their translation [54]. They have been implicated in the regulation of almost all aspects of cellular functions, including the immune responses, innate and adaptive. miRNAs are involved in many types of inflammatory responses and have a significant impact on the magnitude of the responses. Furthermore, they participate of many regulatory networks of genes whose dysfunctions are associated with human diseases such as cancer [55, 56].

The expression of miRNAs is tightly controlled both spatially and temporally. Although some of them may function as tumor suppressors, the aberrant expression of these molecules has been correlated with various types of human cancers [57]. Besides, several miRNAs are involved in many types of inflammatory response. This is done in two main ways: by affecting development of subpopulations of inflammatory cells such as Th2 and Th17, or by setting the level of immune cell function, e.g., controlling the amount of cytokine produced by DCs [58].

Some miRNAs are expressed in activated T lymphocytes, and each miRNA represses its specific targets, which are often transcription factors specific for a given cell line. This may determines the type of inflammatory T cells produced during inflammation. Specific miRNAs, such as miR-155 and miR-146a, expressed in inflammatory cells, have as targets signaling proteins that regulate the intensity of the inflammatory signal. Ideally, the signaling results in a transient inflammatory response that eliminates the infection without harming the host. The lack of certain miRNAs, such as miR-155, can reduce the magnitude of the immune response, resulting in immunodeficiency. On the other hand, the constant overexpression of miR-155 or deletion of miR-146a can cause a chronic inflammatory condition in which inflammation is not resolved [59].

The expression of miR-21, miR-155, and miR125b is controlled by an undetermined amount of immune signals, the most prominent being TLR, TNF- α , and other cytokines that bind the functions of these miRNAs with inflammatory events [60]. The inflammation modulates the expression of microRNAs that influence the production of several tumor-

related messenger RNAs or proteins. These molecular events induced by chronic inflammation contributes to alter important pathways involved in normal cellular function, and hence strengthen the role of inflammation in cancer development [61]. miR-21 is unregulated, both in vitro and in vivo, by oncogenes RAS or SRC, the most frequently activated in human cancers [62].

Among the mechanisms used by miRNAs to promote the initiation and progression of tumors are those that affect the modulation of TLR, cytokines, and their signaling pathways, they also play an important role in the development of cancers associated with infectious agents. The infections with various pathogens induce changes in the expression of miRNAs functionally related to the mounting of the innate immune response. Thus, they are involved in the regulation of the survival and proliferation of immunocompetent cells responsible for the control of infections. The miRNAs miR-21, miR-125, and miR-155 are the most frequently expressed during infection and therefore have a potential role in carcinogenesis induced by infectious agents. It has been shown that overexpression of miR-21 and miR-182 is associated with carcinogenesis associated with HPV with high oncogenic potential [60, 63].

A recent study identified one inflammatory pathway mediated by microRNA that is epigenetically repressed in breast cancers. A high-throughput screen for signal transducer and activator of transcription 3 (STAT3)-regulated microRNAs revealed the microRNA miR-146b as a direct STAT3 target in mammary epithelial cells, but DNA methylation in its promoter area suppressed miR-146b expression in cancer cells. It was observed that deregulated expression of miR-146a and miR-155, facilitates the development of proinflammatory phenotype of Tregs via increased STAT1 activation [64]. Overexpression of miR-146b suppresses NF- κ B in an IL-6-dependent manner. The subsequent STAT3 activation decreased invasiveness phenotype in breast cancer cells [65, 66]. It has been proposed that carcinogenesis induced by inflammatory response triggered by miRNA, in colon cancer is related to dysregulation of colon cells and leukocytes, with impact on proteins involved in the PI3K/Akt signaling pathway, thereby contributing to cancer cell proliferation and tumor growth [67].

Conclusions

Chronic inflammation arising of infections or of autoimmune disease precedes development of tumors, suggesting that inflammatory response plays an important role in the tumorigenesis process. Studies show that chronic inflammation can contribute to initiation, promotion, growth, and invasion of tumors, through of oncogenes activation, induction of mutations, loss of the mechanisms of cell cycle control, and of DNA repair, generating a genomic instability which, together

with angiogenesis and tissue remodeling, contributes to development about 1 in 4 cases, of cancer. The mediators of inflammatory response coordinates the central signaling pathways of activation of the innate and adaptive immune responses, and affect various aspects of inflammation, by activating involved genes in survival and proliferation of cells. Also promotes processing the extracellular matrix proteins and other related signaling molecules, causing abnormal angiogenesis and remodeling of vascular tissue, facilitating recruitment and activation or suppression cells of the immune system. Thus, a large variety of inflammatory mediators act together through a complex network of communication through which, they interact with each other's, of synergistic or antagonistic way, to break the cellular homeostasis, creating favorable conditions for initiation, progression and invasion of tumors. Understanding the mechanisms involved in activation, migration and infiltration of immune cells into tumors, as well as the role of a range of mediators of inflammation in the crosstalk of the immune cells with cancer cells, and the molecular events that mediate this dialog, is of great importance to find ways of intervene in this complex network of events, in order of prevent or interrupt the process of tumorigenesis.

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Exhibit 105

RESEARCH ARTICLE

Inflammation-Induced Cell Proliferation Potentiates DNA Damage-Induced Mutations *In Vivo*

Orsolya Kiraly^{1,2}, Guanyu Gong¹, Werner Olipitz¹, Sureshkumar Muthupalani³, Bevin P. Engelward^{1,2*}

1 Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States of America, **2** Singapore–MIT Alliance for Research and Technology, Singapore, **3** Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States of America

* bevin@mit.edu



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Abstract

Mutations are a critical driver of cancer initiation. While extensive studies have focused on exposure-induced mutations, few studies have explored the importance of tissue physiology as a modulator of mutation susceptibility *in vivo*. Of particular interest is inflammation, a known cancer risk factor relevant to chronic inflammatory diseases and pathogen-induced inflammation. Here, we used the fluorescent yellow direct repeat (FYDR) mice that harbor a reporter to detect misalignments during homologous recombination (HR), an important class of mutations. FYDR mice were exposed to cerulein, a potent inducer of pancreatic inflammation. We show that inflammation induces DSBs (γ H2AX foci) and that several days later there is an increase in cell proliferation. While isolated bouts of inflammation did not induce HR, overlap between inflammation-induced DNA damage and inflammation-induced cell proliferation induced HR significantly. To study exogenously-induced DNA damage, animals were exposed to methylnitrosourea, a model alkylating agent that creates DNA lesions relevant to both environmental exposures and cancer chemotherapy. We found that exposure to alkylation damage induces HR, and importantly, that inflammation-induced cell proliferation and alkylation induce HR in a synergistic fashion. Taken together, these results show that, during an acute bout of inflammation, there is a kinetic barrier separating DNA damage from cell proliferation that protects against mutations, and that inflammation-induced cell proliferation greatly potentiates exposure-induced mutations. These studies demonstrate a fundamental mechanism by which inflammation can act synergistically with DNA damage to induce mutations that drive cancer and cancer recurrence.

Author Summary

People with chronic inflammatory conditions have a markedly increased risk for cancer. In addition, many cancers have an inflammatory microenvironment that promotes tumor

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growth. Here, we show that inflammatory infiltration synergizes with tissue regeneration to induce DNA sequence rearrangements *in vivo*. Chronically inflamed tissues that are continuously regenerating are thus at an increased risk for mutagenesis and malignant transformation. Further, rapidly dividing tumor cells in an inflammatory microenvironment can also acquire mutations, which have been shown to contribute to drug resistance and disease recurrence. Finally, inflammation-induced tissue regeneration sensitizes tissues to DNA damaging environmental exposures and chemotherapeutics. The work described here thus increases our understanding of how inflammation leads to genetic changes that drive cancer formation and recurrence.

Introduction

Effective strategies for preventing and treating cancer depend not only upon understanding genetic and exposure-induced factors, but also physiological factors that drive disease. DNA damage, caused by endogenous metabolites and exogenous agents, promotes mutations, a key driver of phenotypic changes that potentiate metastasis and enable recurrence after treatment [1]. While significant progress has been made in terms of understanding how genes and exposures modulate the risk of mutations, relatively little is known about the potential role of tissue physiology in modulating the risk of mutations *in vivo*. Of particular interest is the inflammatory state, a critical cancer risk factor that is associated with sweeping changes in tissue architecture due to immune cell infiltration and associated changes in the levels of cytokines and reactive oxygen and nitrogen species (RONS) [2–4]. Inflammation is a well-established tumor promoter that contributes to cancer growth, angiogenesis, and resistance to apoptosis [2,5]. In addition to the role of inflammation in cancer progression, it is increasingly recognized that inflammation-induced DNA damage may also drive mutations that contribute to both initiation and progression [3,6]. With recent advances that enable analysis of key factors that impact the risk of mutation [7], here, we set out to determine how interactions between DNA damage and inflammation-induced physiological changes impact the risk of mutations *in vivo*.

It has long been thought that it is the convergence of conditions that induce DNA damage and cell division simultaneously that is a key driver of inflammation-induced mutations [8–11]. Nevertheless, studies that directly query the combined effect of RONS-induced DNA damage and cell division are lacking, both *in vitro* and *in vivo*. Importantly, the same proposed mechanism for synergy between cell division and endogenous RONS applies to exogenous DNA damaging agents. In the clinic, virtually all cancer patients are exposed to high levels of DNA damage when treated with radiation and/or chemotherapy, for which DNA damage is often critical to the mode of action. It is well established that an increase in the mutation rate contributes to cancer promotion and drug resistance [12–15]. Therefore, understanding physiological factors that modulate susceptibility to therapy-induced mutations could open doors to strategies to reduce disease recurrence.

Pancreatic inflammation is a key risk factor for pancreatic cancer [11,16], one of the most deadly cancers; most patients who initially respond to radio-chemotherapy suffer relapse, such that only ~5% of patients survive more than 5 years after diagnosis [17]. Inflammation-induced DNA damage potentially plays an important role in driving mutations that enable pancreatic cancer initiation and recurrence. During inflammation there are high levels of RONS, which can induce cytotoxic and mutagenic DNA lesions, including abasic sites, oxidized bases (e.g., 8oxoG), deaminated bases (e.g., uracil and hypoxanthine) and ethenoadenine (eA) [18,19]. In addition to base damage, RONS also induce DNA double strand breaks (DSBs).

DSBs are among the most toxic of DNA lesions and they can also be potentially mutagenic due to the potential loss of vast stretches of chromosomes if not accurately repaired [1,20].

Homologous recombination (HR) plays a critical role in preventing DSB-induced cytotoxicity by repairing DSBs during S/G₂ [21]. To initiate repair, the DNA is resected by MRE11 and EXO1 to generate 3' single-stranded overhangs [22–25]. BRCA2 then loads RAD51 onto the single-stranded DNA to form a nucleoprotein filament capable of homology searching and strand invasion [26–30]. The resulting D-loop enables the copying of sequence information that can then be processed by downstream proteins to complete the repair process [21]. While HR is effective for repair of two-ended DSBs, its most important role is in the repair of one-ended DSBs that arise when replication forks break down. Unlike two-ended DSBs, which can be repaired by alternative mechanisms, one-ended DSBs require HR for accurate sequence realignment and reinsertion of the broken DNA end. Inflammation induces single strand breaks and replication-blocking lesions, both of which promote replication fork breakdown. Furthermore, mutations in BRCA2 are genetic risk factor for pancreatic cancer [31], indicating that HR is indeed active in the pancreas [32]. Thus, RONS are predicted to create DSBs during pancreatitis, and HR can potentially repair inflammation-induced DSBs in the pancreas.

Ironically, while HR prevents cytotoxicity and is mostly accurate, HR carries a risk of sequence changes. Misalignments during HR promote large scale sequence rearrangements, such as deletions, duplications and translocations [33–35], and these HR-driven events have been observed in cancers [36,37]. Furthermore, HR between homologous chromosomes can also lead to loss of heterozygosity (LOH), a major mechanism for the inactivation of tumor suppressor genes. Indeed, studies with cultured cells have demonstrated that HR is the underlying cause of 30 to 70% of LOH events [38–40], and the importance of HR-driven LOH has also been demonstrated in tumors [41,42]. Finally, it has recently been shown that HR also promotes point mutations in mammalian cells, due to misincorporation during repair synthesis [43–48]. Taken together, it is now clear that virtually all cancers harbor one or more HR-driven sequence changes that promote initiation and progression.

Given the importance of HR, we created a mouse model that enables the detection of HR *in vivo* (see ref. 7). The fluorescent yellow direct repeat (FYDR) mice harbor an integrated direct repeat comprised of two non-functional EYFP expression cassettes, wherein transfer of sequence information by HR from one cassette to the other can reconstitute full length sequence and give rise to fluorescence (Fig. 1A–D) [49]. The FYDR recombination substrate is designed to detect the major classes of HR events, including gene conversion (wherein sequence information is transferred from one duplex to the other), sister chromatid exchange (e.g., gene conversion with crossover) and replication fork repair (S1 Fig.) [50]. Importantly, FYDR fluorescence after replication fork repair indicates misalignment and transfer of sequence information during HR, and in some cases the gain of one repeat unit in the FYDR substrate (Fig. 1A). Given that all cells that are positive for fluorescence result from sequence misalignment and harbor a change in sequence information, the FYDR readout is indicative of mutation events. The FYDR mouse model thus affords key advances in studies of mutagenesis, since it became possible for the first time to visualize mutant cells that arise within intact tissues of adult animals [7].

Here, we have integrated approaches for visualization and quantification of DNA damage, cell proliferation, and mutation within intact tissues in order to learn about their interrelationships in the context of inflammation. We found that following controlled induction of acute inflammation, the timing for inflammation-induced DSBs is separate from the timing for cell proliferation, creating a protective kinetic barrier against potential synergy between DNA damage and cell division. Breaking this barrier by creating overlap between peak cell proliferation and the acute phase of inflammation causes a synergistic increase in HR-driven mutations.

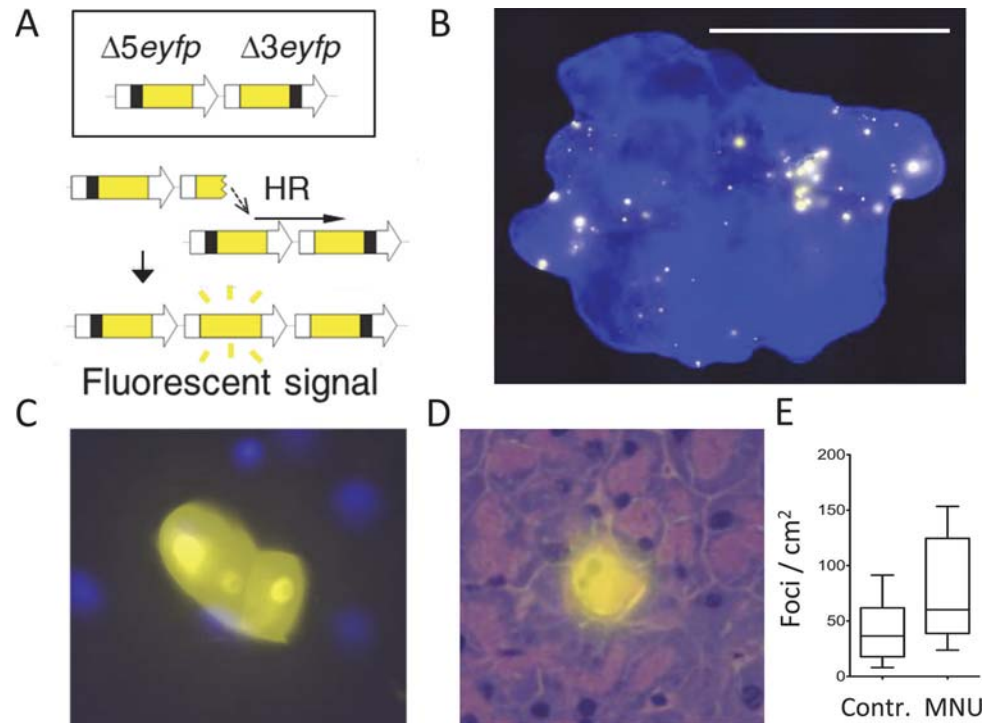


Figure 1. The FYDR mouse detects HR-derived sequence rearrangements *in situ* in intact tissue. (A) Schematic of the reconstitution of full-length EYFP coding sequence from two truncated copies through replication fork restart by HR. Note that the appearance of fluorescent signal indicates the gain of one repeat unit (a duplication). Arrows represent expression constructs. EYFP coding sequences are in yellow, promoter and polyadenylation signal sequences are in white, and deleted sequences are in black. Drawing is not to scale. (B) Representative image of a FYDR pancreas showing fluorescent foci detectable *in situ* in intact tissue. Freshly harvested, unfixed whole pancreas was counterstained with Hoechst, compressed to 0.5 mm and imaged under an epifluorescent microscope. Fluorescence is pseudocolored. Original magnification, $\times 1$. Scale bar = 1 cm. (C) Cluster of recombinant cells at $\times 60$ original magnification. Fluorescence is pseudocolored. (D) A recombinant pancreatic acinar cell identified by the overlay of EYFP fluorescence and H&E staining. Fluorescence is pseudocolored. Original magnification, $\times 40$. (E) The model alkylating agent MNU induces HR in the pancreas. Mice received 25 mg/kg MNU i.p., and HR was evaluated 3 to 5 weeks after treatment. Frequencies of recombinant foci per cm² tissue area are significantly greater in MNU-treated mice ($n = 15$) than in control mice ($n = 16$). Boxes show 25th and 75th percentiles, medians are indicated by horizontal lines. * $P < 0.05$ (Mann–Whitney *U*-test).

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Furthermore, under conditions of inflammation-induced cell proliferation, there is a dramatic increase in susceptibility to mutations induced by exposure to an exogenous DNA damaging agent of a class that is present in environmental contaminants and also commonly used in the clinic. This work reveals the critical role that tissue physiology plays in mutation susceptibility and opens doors to new avenues of cancer prevention and treatment.

Results

FYDR mice enable studies of DNA damage-induced HR

In the FYDR mice, HR-induced misalignments between two copies of an expression cassette for EYFP are detectable as fluorescent foci within intact pancreatic tissue (Fig. 1A,B). In some cases, foci are comprised of more than one fluorescent recombinant cell, indicative of a recombination event in a single cell that has subsequently undergone clonal expansion (Fig. 1C) [51]. Analysis of tissue histology shows that in the pancreas, acinar cells undergo HR (Fig. 1D), and

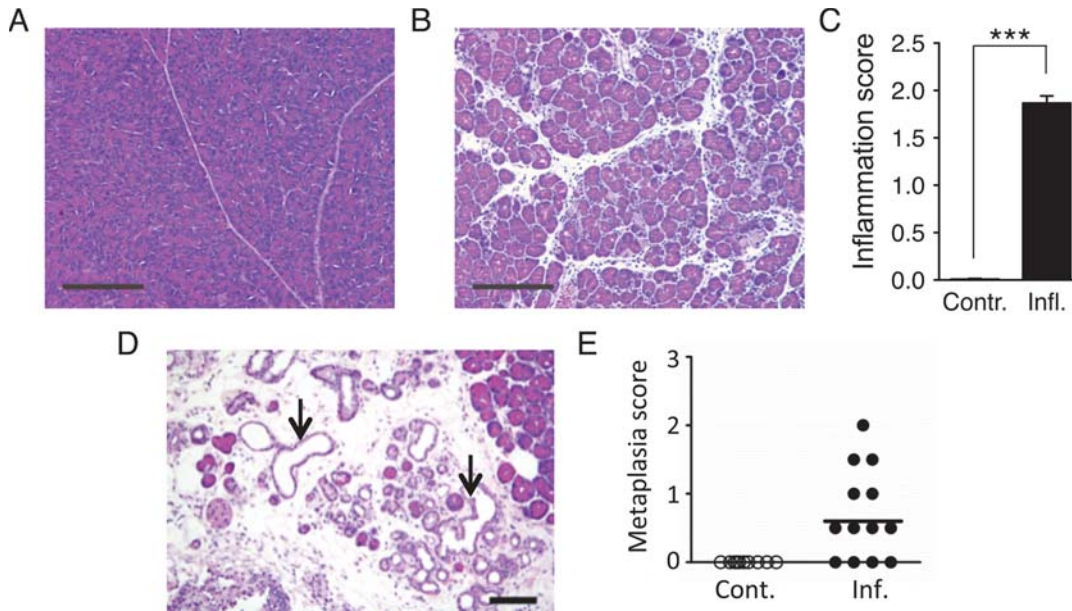


Figure 2. Cerulein treatment induces inflammation in the pancreas, and chronic cerulein pancreatitis induces metaplastic changes. (A) Tissue sections from pancreata of control mice show normal pancreas architecture. (B) Acute cerulein treatment induces pancreatic inflammation evidenced by edema and an inflammatory infiltrate. (C) Severity of cerulein-induced inflammation as determined by a trained pathologist. Inflammation scores are significantly higher in cerulein-treated mice ($n = 30$) than in control mice ($n = 30$). Data are mean \pm SEM. *** $P < 0.001$ (Student's t -test). (D) Pancreas section from a mouse treated with cerulein for 6 months shows chronic pancreatic inflammation, edema, significant acinar loss, and acinar to ductal metaplasia (arrows). (E) Quantification of metaplastic changes determined by a trained pathologist shows absence of metaplasia in control mice. However, 9 out of 13 mice treated with cerulein for 6 months show metaplastic changes. See [Methods](#) for detailed pathological scoring criteria. Statistical testing could not be performed in groups containing only zero values. Panels A,B: Original magnification, $\times 10$. Scale bar = $200 \mu\text{m}$. Panel D: Original magnification, $\times 200$. Scale bar = $80 \mu\text{m}$.

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previous studies show that acinar cells comprise virtually all of the recombinant cells in the FYDR pancreas [51].

The FYDR mice enable studies of exposure-induced HR in the pancreas of adult animals. Of particular interest are alkylating agents, an important class of DNA damaging agents that are present in food and in our environment, some of which have been shown to cause cancer [52–54]. Ironically, alkylating agents are used to treat cancer when given at high doses [55]. Temozolomide, a methylating agent that is used in cancer chemotherapy, kills tumor cells by creating DNA lesions that either directly or indirectly inhibit DNA replication, causing cytotoxicity [55]. Cells that do not die from exposure to temozolomide potentially run the risk of harboring chemotherapy-induced mutations, including HR events. To determine if alkylation damage induces HR in the pancreas, FYDR mice were exposed to the model methylating agent MNU, which creates the same types of base lesions as temozolomide. Results show that MNU causes a significant increase in the frequency of fluorescent foci (Fig. 1E), indicating that the FYDR mouse model is effective for studies of DNA damage-induced HR.

Pancreatic inflammation induced by cerulein leads to edema and precancerous lesions

In order to study the interactions between DNA damage and inflammation, we exploited cerulein, a cholecystokinin analog that is well established as an inducer of pancreatic inflammation [56,57]. Animals exposed to cerulein by 6 hourly intraperitoneal injections showed pancreatic edema and infiltration by inflammatory cells, chiefly neutrophils (Fig. 2A,B). The extent of features of pancreatitis was found to be statistically significantly increased when quantified by a

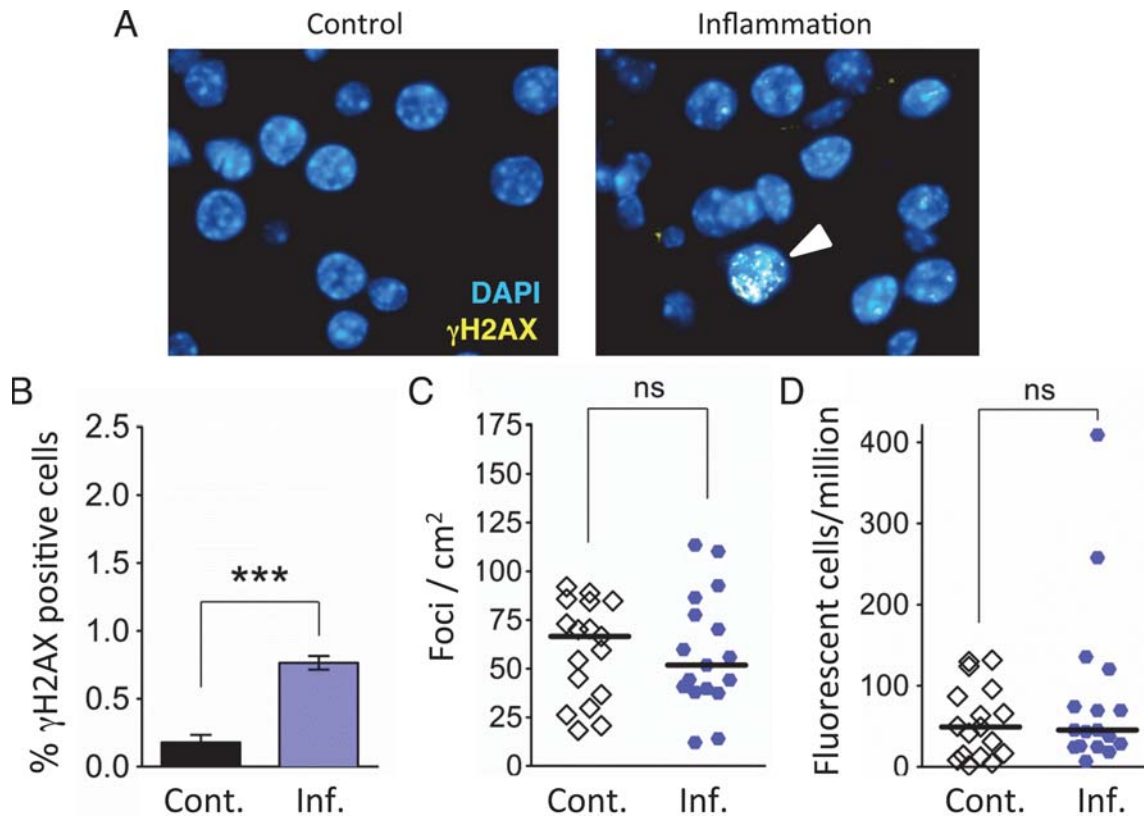


Figure 3. Independent bouts of inflammation induce DSB formation but not HR. (A) Immunohistochemical staining for the DSB marker γ H2AX (yellow) in pancreas sections. Nuclei were counterstained with DAPI (blue). In control mice, nuclei with γ H2AX foci are very rare (Left). However, nuclei with γ H2AX foci (arrowhead) appear after independent bouts of inflammation (Right). (B) Quantification of nuclei containing more than five γ H2AX foci shows significantly more γ H2AX positive nuclei after inflammation ($n = 6$) than in control animals ($n = 6$). Data are mean \pm SEM. *** $P < 0.001$ (Student's t -test). (C) Numbers of fluorescent foci in the pancreas are not different between control mice ($n = 17$) and mice that underwent repeated acute inflammation ($n = 17$). Symbols represent data from individual mice, horizontal bars show medians. ns, not statistically significant (Mann-Whitney U -test). (D) No statistically significant difference in the frequencies of fluorescent cells in the pancreas between control mice ($n = 17$) and mice that underwent repeated acute inflammation ($n = 17$). Pancreata were disaggregated into single-cell suspensions and the frequencies of fluorescent cells were determined by flow cytometry. Symbols represent data from individual mice, horizontal bars show median values. ns, not statistically significant (Mann-Whitney U -test).

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trained pathologist (Fig. 2C). In studies of long term exposure to cerulein, we observed severe tissue atrophy and metaplasia in wild type mice (Fig. 2D,E), and precancerous lesions in K-Ras mice (S2 Fig.), indicating that cerulein exposure serves as a relevant model for pancreatitis-induced cancer.

Acute inflammation induces DSBs

During inflammation, an increase in the levels of macrophages and neutrophils leads to increased levels of RONS [18]. RONS in turn induce base lesions including eA, 8oxoG and Hx, which have been observed at sites of inflammation [18,19]. Many RONS-induced DNA lesions have the potential to cause recombinogenic DSBs through chemical cleavage, by enzymatic processing, or as a result of replication fork breakdown [58–60]. To learn if pancreatic inflammation induces DSBs *in vivo*, we analyzed the frequency of DSB repair foci by quantifying cells with five or more γ H2AX foci (H2AX becomes phosphorylated to form γ H2AX in the vicinity of DSBs) [61]. Immunohistochemical (IHC) analysis of pancreatic tissue reveals a clear induction of DSBs after exposure to cerulein (Fig. 3A,B).

Repeated exposure to acute inflammation does not cause a detectable increase in HR

As HR has been shown to be induced by DSBs *in vitro* [62,63], we next asked if DSBs associated with acute inflammation induce HR *in vivo*. To increase the sensitivity of our approach, animals were exposed to three bouts of acute pancreatitis. Analysis of the frequency of HR events in control animals shows that there is variation in the frequencies of foci/cm², ranging from ~15 to ~100 (Fig. 3C), consistent with previous studies [7,64,65]. (It is noteworthy that variation in mutation frequency among normal animals has similarly been shown in several other mouse models for mutation detection [66–69]). Unexpectedly, in animals that were subjected to three bouts of inflammation, we did not detect any increase in the frequency of recombination events (indicated by fluorescent foci; Fig. 3C). Analysis of the frequency of fluorescent recombinant cells similarly did not reveal any increase in HR in the animals exposed to three bouts of inflammation (Fig. 3D).

Inflammation-induced cell proliferation occurs days after infiltration and edema

HR is active during S/G₂, whereas most cells in healthy pancreatic tissue are non-dividing cells in G₀/G₁ [70], raising the possibility that HR was not active in RONS-exposed cells during the three bouts of inflammation. To learn about the extent of cell division during the course of inflammation, we quantified dividing cells when tissue is healthy (Fig. 4A), subject to acute inflammation (Fig. 4B) or recovering (Fig. 4C; five days after cerulein exposure, when features of inflammation have cleared). Cell proliferation during the course of the inflammatory response was evaluated by staining for Ki-67, a marker of cell proliferation [71]. Results show that there are very few Ki-67 positive cells in control and acutely inflamed tissue (Fig. 4D,E). In contrast, the frequency of Ki-67 positive cells is significantly induced during tissue recovery (Fig. 4F) and when quantified using image analysis software (see [Materials and Methods](#)) (Fig. 4G). As an alternative approach, animals were treated with BrdU, a thymidine analog that becomes integrated into the DNA of dividing cells and can be detected using immunohistochemistry. Pancreatic tissue was disaggregated, and the frequency of BrdU positive cells was analyzed by flow cytometry. Consistent with the Ki-67 analysis, results show a clear increase in the frequency of dividing cells several days after acute inflammation (Fig. 4H). Thus, with both methods, we found that acute phase inflammation is separate from a subsequent proliferative phase.

Creating overlap between the acute and proliferative phases of inflammation causes sequence rearrangements

As HR is active primarily during S/G₂, we hypothesized that the lack of HR induction following three independent bouts of inflammation might be due to the kinetic separation between acute inflammation-induced DSBs and recovery-induced cell proliferation. We therefore asked if inflammation might induce HR if the timing were adjusted to create overlap between inflammation-induced DSBs and cell proliferation. For ‘protocol 1’ described above, animals were exposed to three independent bouts of inflammation, each two weeks apart (Fig. 5A). Here, for ‘protocol 2’, animals were also exposed to three bouts of inflammation, however bouts of inflammation were 4–5 days apart (Fig. 5B).

For ‘protocol 1’, we observed that exposure to cerulein induces acute inflammation, as can be seen by the edema and infiltration under inflamed conditions (compare Fig. 5C and 5D). At the time of acute inflammation, the frequency of dividing cells is unchanged compared to untreated animals (Fig. 5F,G). However, cells with high numbers of γ H2AX foci are apparent

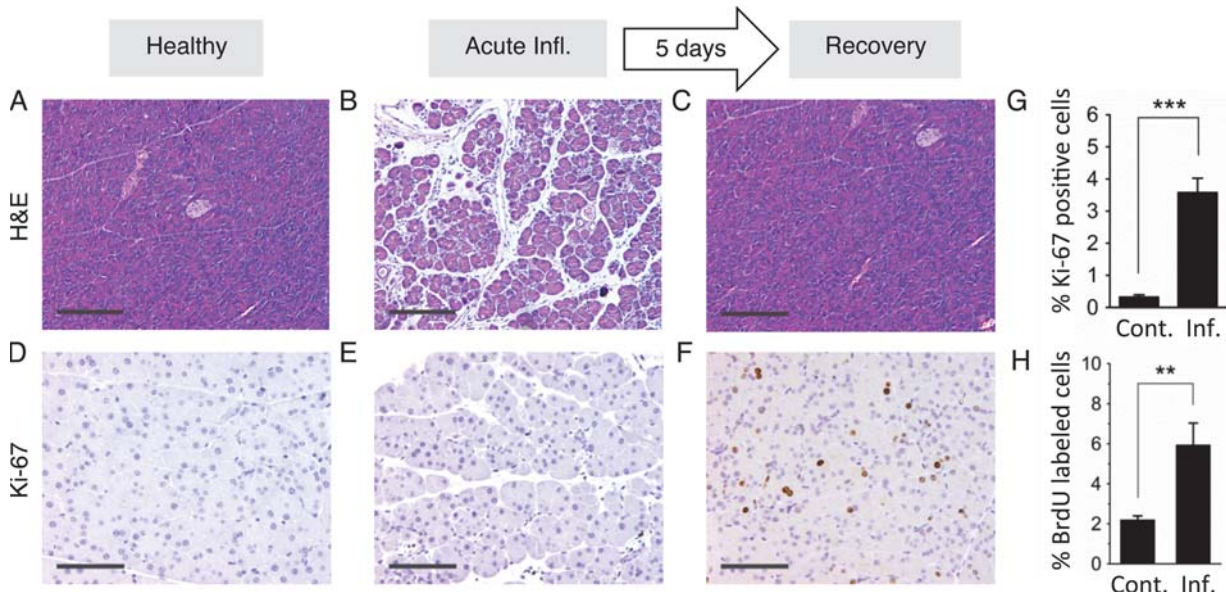


Figure 4. Inflammation and regenerative cell proliferation are separated in acute cerulein pancreatitis. (A) Pancreas from control mouse showing normal tissue architecture with no detectable histological changes. (B) 12 hours after acute cerulein treatment, the pancreas shows histological signs of acute pancreatitis, such as edema and an inflammatory infiltrate. (C) Five days after acute cerulein treatment, inflammation is no longer detected and histology is comparable to healthy tissue. (D) Low Ki-67 staining indicates low proliferative activity in control pancreata. (E) Ki-67 staining remains low during acute pancreatitis, indicating no increase in cell proliferation during acute inflammation. (F) Five days after acute cerulein treatment, increased Ki-67 staining indicates increased cell proliferation during tissue regeneration. (G) Quantification of Ki-67 labeling shows significantly higher proliferation in regenerating tissue. Data are mean \pm SEM in control mice ($n = 16$) and in mice with acute pancreatitis ($n = 16$). *** $P < 0.001$, Student's t -test. (H) Increased cell proliferation during regeneration from acute pancreatitis is indicated by increased BrdU labeling. Five days after acute pancreatitis or mock treatment, mice received BrdU (75 mg/kg i.p.) to label newly replicated DNA in proliferating cells. Pancreata were harvested 4 hours later, disaggregated, and the frequencies of BrdU labeled cells were determined by antibody staining and flow cytometry. Data are mean \pm SEM in control mice ($n = 5$) and in mice with acute pancreatitis ($n = 5$). ** $P < 0.01$, Student's t -test. Panels B,C,D: Original magnification, $\times 10$. Scale bar = 200 μ m. Panels E,F,G: Original magnification, $\times 20$. Scale bar = 100 μ m.

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(Fig. 5J), which is consistent with DNA damage formed by RONS that are associated with the acute phase of inflammation. We also observed acute inflammation using 'protocol 2' (Fig. 5E). Unlike protocol 1, we also observed concomitant induction of cell division, consistent with the proliferative phase of the first bout of inflammation (Fig. 5H). Cells with high frequencies of γ H2AX foci are evident (Fig. 5K).

To learn more about the impact of overlap between bouts of inflammation, the extent of inflammation was assessed by a trained pathologist, the extent of cell proliferation was quantified by automated image analysis, and the frequency of γ H2AX positive cells was measured by counting cells with >5 γ H2AX foci. Results show that the severity of the acute phase of inflammation is similar regardless of whether bouts of inflammation occur independently or in an overlapping fashion (Fig. 6A,B). In contrast, cell proliferation is dramatically increased under conditions where the response to the first bout of inflammation overlaps with initiation of the second bout of inflammation (Fig. 6C,D). The frequency of DSBs is increased in both independent and overlapping bouts of inflammation, and the increase is greater under conditions of overlap between the acute phase of inflammation and the proliferative phase (compare Fig. 6E and Fig. 6F). Similar results were observed for the third bout of inflammation (S3–S4 Fig.), although the frequencies of γ H2AX were reduced during the third bout of inflammation relative to the second bout under conditions of overlap. It is unclear why the third bout of inflammation is apparently less damaging, however one possibility is that HR proficiency increased during the course of the exposure protocol, leading to more rapid clearance of DSBs. It is

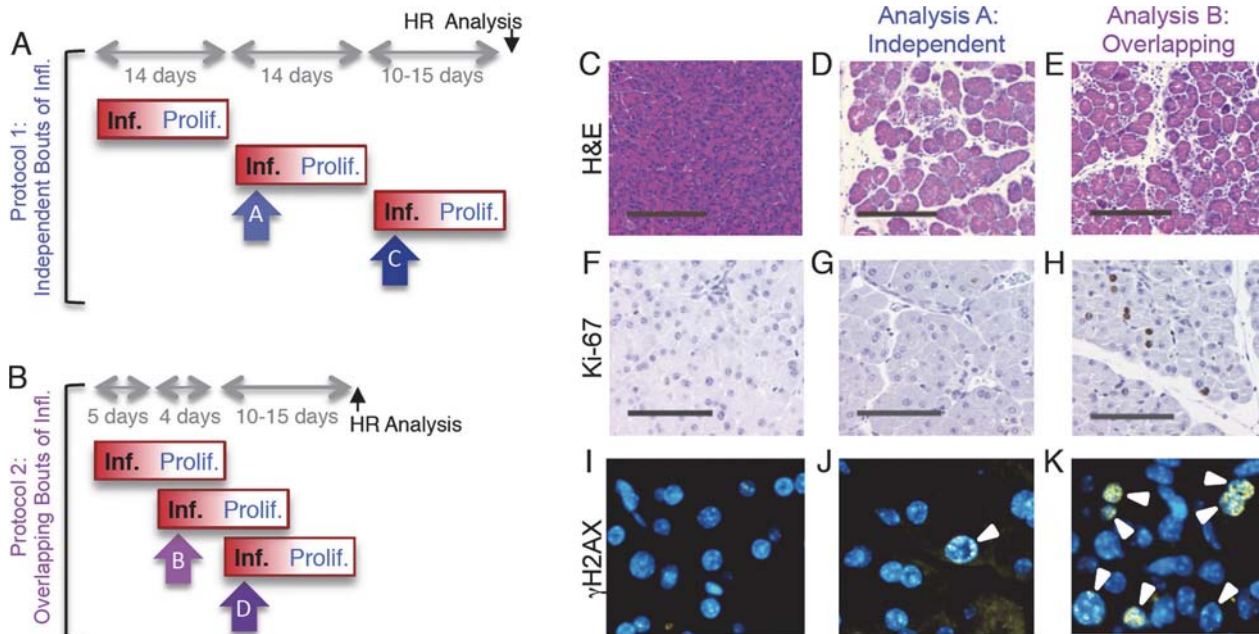


Figure 5. Independent and overlapping bouts of pancreatic inflammation. (A) For independent bouts of inflammation, three acute cerulein pancreatitis events were induced two weeks apart, and inflammation and proliferation were assessed at the second (analysis time A) and third (analysis time C) bout of inflammation. HR was quantified 10 to 15 days after the last pancreatitis event. (B) For overlapping bouts of inflammation, three acute cerulein pancreatitis events were induced on days 1, 4 and 9. Inflammation and proliferation were assessed at the second (analysis time B) and third (analysis time D) bout of inflammation. HR was quantified 10 to 15 days after the last pancreatitis event. (C) Pancreas section from a control mouse shows healthy tissue. (D,E) Treatment with cerulein (both independent and overlapping) results in edema and an inflammatory infiltrate chiefly of neutrophils, indicating acute inflammation. (F) Ki-67 immunohistochemistry shows low levels of baseline proliferation in control pancreata. (G) Cell proliferation remains low in the pancreas during acute inflammation. (H) During regeneration from acute inflammation, Ki-67 positive nuclei appear, indicating regenerative proliferation. (I) Immunohistochemical detection of γ H2AX phosphorylation in pancreas sections show low levels of DSBs in healthy pancreata. (J) During independent bouts of inflammation, nuclei with γ H2AX foci (arrowhead) become apparent. (K) During overlapping bouts of inflammation, more γ H2AX positive nuclei are visible. (C-E) Original magnification, $\times 10$. Scale bar = 200 μ m. (F-H) Original magnification, $\times 20$. Scale bar = 100 μ m. (I-K) Original magnification, $\times 40$.

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noteworthy that clearance of potentially toxic DSBs is advantageous to cell survival, but carries the risk of mutations due to HR misalignments.

To learn about the impact of inflammatory response kinetics on susceptibility to HR, recombination events were quantified within intact pancreatic tissue, and the frequency of recombinant cells was evaluated in disaggregated pancreatic tissue by flow cytometry. Under conditions of overlapping bouts of inflammation (protocol 2), there is a significant increase in the frequency of recombination events, which is both visually apparent (Fig. 7A) and quantitatively significant (Fig. 7B). In addition, there is a significant increase in the frequency of fluorescent recombinant cells under conditions of overlap (Fig. 7C), but not when animals are exposed to three independent bouts of inflammation (Fig. 3D).

Inflammation potentiates rearrangements induced by a model cancer chemotherapeutic

The observation that overlapping bouts of inflammation induce HR is consistent with a model wherein inflammation-induced cell proliferation sensitizes tissue to HR induced by endogenously-produced DNA damage. We next asked about the potential for inflammation-induced cell proliferation to cause increased sensitivity to HR induced by an exogenous DNA damaging agent, specifically the model cancer chemotherapeutic, MNU.

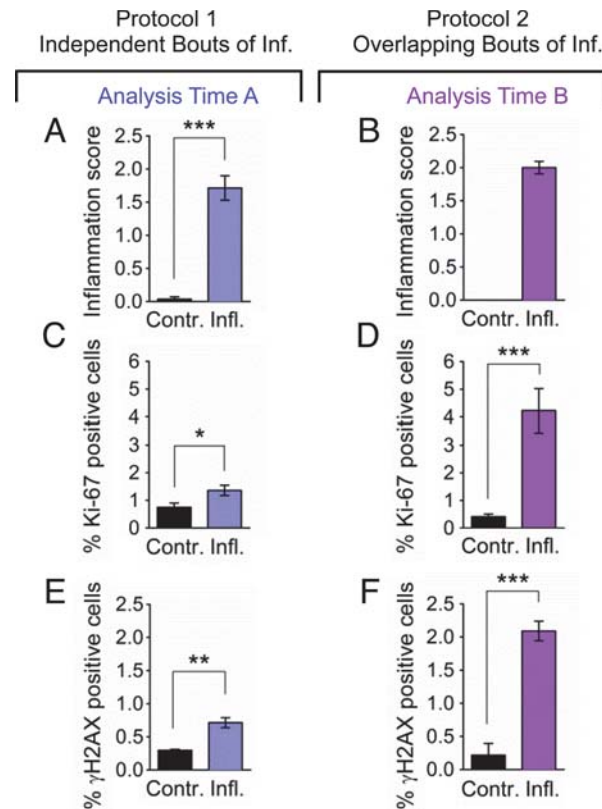


Figure 6. Overlapping bouts of inflammation induce more DSBs than independent bouts of inflammation. Inflammation, cell proliferation and γH2AX foci formation were quantified in pancreas sections from mice treated with independent bouts of inflammation (blue bars) and with overlapping bouts of inflammation (purple bars). (A,B) Cerulein induces inflammation in both independent (n = 7) and overlapping (n = 8) treatment regimens. Severity of inflammation in control and cerulein-treated mice was quantified by a trained pathologist. (C, D) Quantification of nuclei positive for the proliferation marker Ki-67 shows a moderate increase in independent bouts of inflammation (n = 7), and a large increase in overlapping bouts of inflammation (n = 8). (E,F) Quantification of nuclei positive for the DSB marker γH2AX (nuclei with >5 foci) shows a moderate increase in independent bouts of inflammation (n = 3), and a large increase in overlapping bouts of inflammation (n = 3). Data are mean ± SEM. See [Methods](#) for detailed pathological scoring criteria. Statistical testing could not be performed in groups containing only zero values. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test).

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Experiments were designed with the objective of finding the time when inflammation-induced cell proliferation is high, and then exposing animals to MNU ([Fig. 8A](#)). To quantify the extent of inflammation-induced proliferation, pancreatic tissue was analyzed for Ki-67 positive cells. There is a significant increase in cell proliferation at the time of the MNU exposure ([Fig. 8B](#)). MNU on its own causes a visually apparent ([Fig. 8C](#)) and statistically significant increase in the frequency of HR events in healthy animals ([Fig. 8D](#)) (note that the data from [Fig. 1E](#) have been replotted to facilitate comparisons among cohorts). The effect of MNU on HR was dose dependent: at 25 mg/kg, there was a statistically significant increase in the number of fluorescent foci ([Fig. 8D](#)), whereas there was not a significant increase in HR after treatment with 7.5 mg/kg MNU ([S5 Fig.](#)). We also found that a single bout of inflammation does not induce HR ([Fig. 8C,D](#)), which is consistent with results shown above ([Fig. 3](#)). Importantly, when animals were exposed to MNU at a time when inflammation-induced proliferation is high, there was a dramatic increase in the frequency of HR ([Fig. 8C,D](#)), revealing that physiological changes associated with inflammation and exposure to an exogenous DNA damaging

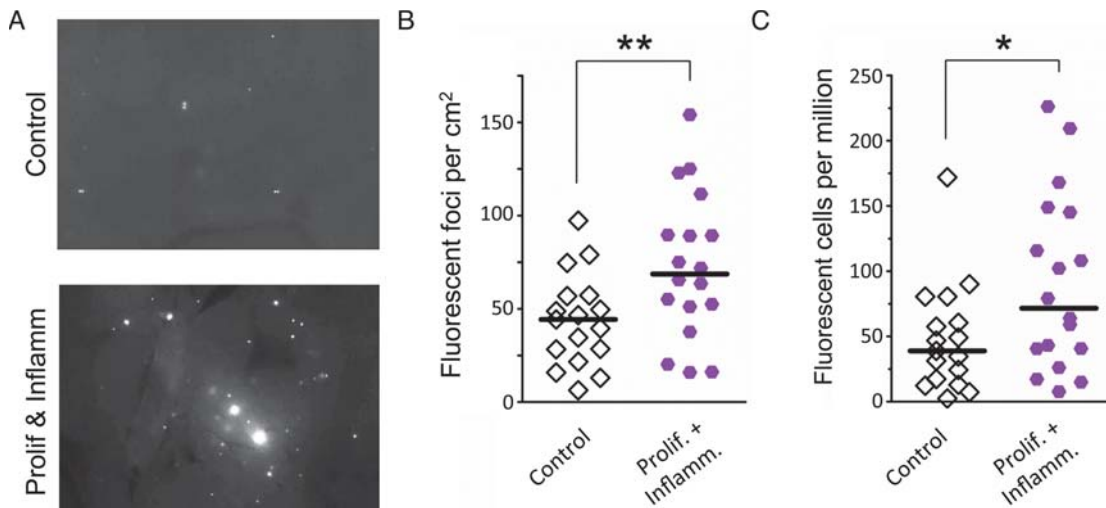


Figure 7. Simultaneous inflammation and cell proliferation induces HR in the pancreas. (A) Representative images from pancreata of control mice (Top) and mice that experienced combined proliferation and inflammation (Bottom). Freshly harvested whole organs were compressed between glass coverslips and imaged under an epifluorescent microscope. Representative details of composite images are shown, fluorescent foci are apparent *in situ*. More foci are visible in the pancreas from the proliferation plus inflammation group. Brightness and contrast have been enhanced identically. (B) Numbers of fluorescent foci are higher in mice that experienced combined proliferation and inflammation ($n = 18$) than in control mice ($n = 17$). Symbols represent data from individual mice, horizontal bars show medians. **, $P < 0.01$, (Mann–Whitney *U*-test). (C) Higher fluorescent cell frequency in the pancreata of mice that experienced combined proliferation and inflammation ($n = 18$) than in control mice ($n = 17$). Pancreata were disaggregated into single-cell suspensions and the frequencies of fluorescent cells were determined by flow cytometry. Symbols represent data from individual mice, horizontal bars show median values. *, $P < 0.05$ (Mann–Whitney *U*-test).

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agent act synergistically to induce HR. These results call attention to the importance of inflammation as a modulator of DNA damage-induced sequence rearrangements induced by exposure to an alkylating agent that serves as a model for environmental and clinical DNA damaging agents.

Discussion

Pancreatic cancer is one of the most deadly cancers, yet relatively few studies have explored factors that govern susceptibility to mutations that initiate pancreatic cancer. Furthermore, while radiation and chemotherapy can be effective initially, recurrence is virtually inevitable [72], and mutations are a key driver of recurrence since they enable evolution into drug resistant and more aggressive phenotypes [12–15]. Thus, there is a need for a deeper understanding of the mechanisms of DNA damage-induced mutations in the pancreas. Furthermore, while it is well established that pancreatitis is a key risk factor for pancreatic cancer [11,16], studies had not previously been done to explore how physiological changes associated with inflammation modulate the risk of mutations *in vivo*. Here, we show that pancreatic inflammation leads to DNA double strand breaks, and that pancreatitis is associated with hyperproliferation. By creating conditions where there is overlap between bouts of inflammation, we show that DSBs and hyperproliferation act synergistically to induce sequence rearrangements *in vivo* (Fig 9), which both demonstrates a correlation between DSBs and HR *in vivo* and provides insights into the underlying mechanisms that make pancreatitis a risk factor for cancer. Furthermore, we show that inflammation-induced proliferation acts synergistically with a DNA alkylating agent to induce sequence rearrangements *in vivo*, providing new understanding into factors that modulate the risk of sequence changes that promote cancer.

For decades, it has been known that inflammation is a risk factor for cancer [11,16], and it has long been postulated that it is the combination of inflammation-induced DNA damage

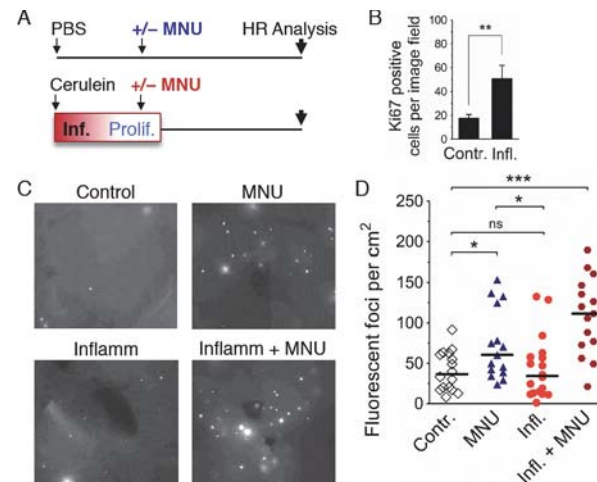


Figure 8. Inflammation-associated cell proliferation potentiates the effect of exogenous DNA damage on DNA rearrangements. (A) Treatment scheme. Mice were subjected to a single acute cerulein pancreatitis event or mock treatment. At the peak of replacement proliferation, mice received MNU (25 mg/kg i.p.) or mock treatment. 3 to 4 weeks after MNU injection, mice were humanely sacrificed for HR analysis. (B) Replacement proliferation in the pancreas is indicated by increased Ki-67 expression. Five days after acute pancreatitis or mock treatment, pancreata were harvested and processed for Ki-67 immunohistochemistry. Data are mean \pm SEM in control mice ($n = 7$) and in mice with acute pancreatitis ($n = 8$). $** P < 0.01$, Student's t -test. (C) Representative images from pancreata after inflammation and/or exogenous DNA damage. Freshly harvested whole organs were compressed between glass coverslips and imaged under an epifluorescent microscope. Representative details of composite images are shown, fluorescent foci are apparent *in situ*. More foci are visible after treatment with MNU, and a large increase is evident after treatment with MNU during regenerative proliferation (Inflamm+MNU panel). (D) Quantification of fluorescent foci in pancreata after inflammation and/or exogenous DNA damage. The number of fluorescent foci is significantly higher in MNU-treated mice ($n = 15$) than in control mice ($n = 16$), but there is no statistically significant increase after a single acute inflammation event ($n = 18$). However, there is a large increase in the number of foci after treatment with MNU during regenerative proliferation (Inflamm + MNU, $n = 15$). Symbols represent data from individual mice, horizontal bars show median values in each group. $*$, $P < 0.05$; $***$, $P < 0.001$ (Mann-Whitney U -test).

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and inflammation-induced cell proliferation that plays a key role in promoting mutagenesis [8–11]. Nevertheless, direct evidence for this model was lacking. Here, we show that, unexpectedly, several bouts of acute inflammation on their own are not sufficient to drive sequence rearrangements, and that separation of the acute phase of inflammation (associated with RONS and DNA damage) and the proliferative stage of inflammation provides a barrier to DNA damage-induced sequence rearrangements. Consequently, conditions that lead to chronic inflammation may be more likely to potentiate tumorigenic mutations compared to isolated bouts of inflammation, which is consistent with epidemiological studies [73,74].

Here, we observed that approximately half of the animals exposed to overlapping bouts of inflammation have frequencies of recombinant cells that are ~ 100 – 200% higher than the untreated control animals. Given that the mutation rate can be rate limiting in tumor promotion [14], a doubling of the mutation frequency could potentially double the probability of cancer recurrence. An increased risk of mutations has relevance to many medical conditions that are associated with chronic inflammation [4]. Inflammatory bowel diseases such as ulcerative colitis and Crohn's disease involve chronic inflammation in the colon, while chronic esophagitis and pancreatitis affect the upper gastrointestinal tract and the pancreas respectively. In addition, chronic infections with bacteria, viruses and parasites can lead to chronic inflammation at multiple sites. Importantly, chronic inflammatory conditions typically last for an extended

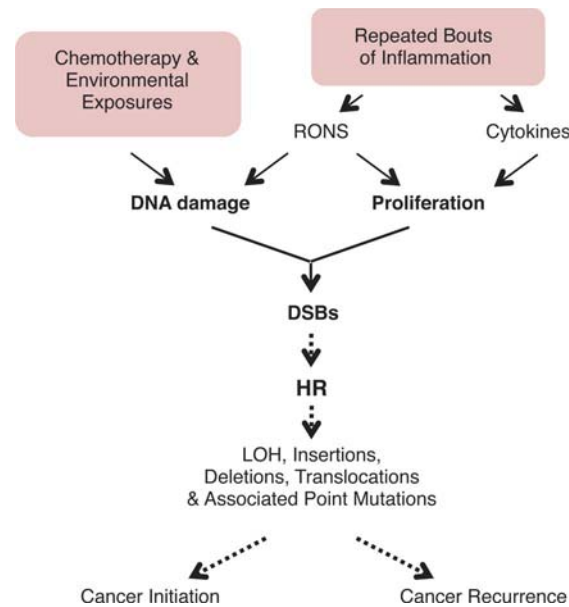


Figure 9. Model for the potentiation of sequence rearrangements induced by endogenous and exogenous DNA damage by inflammation-associated cell proliferation. Cell proliferation associated with inflammation may be induced by RONS released from inflammatory cells. Regeneration after inflammation also involves cell proliferation to replenish cells lost to inflammation-induced tissue damage. DNA replication is increased in proliferation, and DNA damage during replication can lead to fork breakdown and the formation of DSBs. These DSBs are repaired by HR, but HR can result in LOH, sequence rearrangements, and point mutations. Thus, cell proliferation potentiates the deleterious effect of both endogenous (RONS-induced) and exogenous (exposure-induced) DNA damage, potentially contributing to cancer initiation and recurrence. See text for details.

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period of time. Thus, a relatively small increase in susceptibility to mutations in people is anticipated to become very significant given the accumulation over a period of years.

RONS create a wide array of DNA lesions that includes dozens of different types of base lesions as well as abasic sites and strand breaks [75–77]. There is a wealth of information about the mutagenicity of RONS-induced DNA damage derived from studies *in vitro* [78,79]. Many elegant studies have revealed the mutagenic potential of specific RONS-induced base lesions using site-specific lesion technology [80], and many others have described the ability of inflammatory chemicals to induce mutations in RONS-exposed cells *in vitro* [81,82]. Using these and other approaches, we now know quite a lot about the molecular and biochemical mechanisms of RONS-induced mutagenesis. For example, 8oxoG readily mispairs with thymine when bypassed by translesion polymerases [83,84], and that cells prevent TLS-driven mutagenesis by removing 8oxoG [85–87]. Cells also have additional strategies for preventing RONS-induced mutations, including removal of damaged bases from the nucleotide pool (e.g., Mth1) [88,89], and removing the misincorporated base opposite the lesion post replication (e.g., removal of adenine across from 8oxoG by Mutyh) [89, 90]. While the literature describing RONS-induced base lesions *in vitro* is extensive (we refer the reader to several excellent reviews [78,79,81,82]), relatively few studies have addressed RONS-induced mutagenesis *in vivo*. These studies showed that base excision repair is critical in suppressing RONS-induced mutations *in vivo* [91–94], and that inflammation induces mutations in the affected tissues [95–97]. Interestingly, in one such study it was shown that *H. pylori* infection is associated with mutations [98], however the frequency of mutations decreased when Ogg1 was knocked out, leaving unclear the mechanism of mutagenesis. In another study, Ogg1 was found to suppress mutations induced

by oxidative damage [99]. The most direct evaluation of the relationships among inflammation, DNA damage, mutagenesis, and cancer was done recently in the laboratory of L. Samson. This study showed that a deficiency in the Aag glycosylase is associated with increased inflammation-induced cancer, and that tumors harbor mutations consistent with the predicted mutations that would result from an Aag deficiency [6].

Here, we have extended the *in vivo* studies of inflammation and mutagenesis to specifically query the inter-relationships among inflammation, cell proliferation, DSBs, and their consequences (homologous recombination events), using tools that had not previously been applied to this problem. It is important to note that this study focuses on a specific class of mutation (HR-driven sequence rearrangements), and that there are other classes of mutations that are not detected by the FYDR assay, such as base damage-induced point mutations (which often arise during TLS), and small insertions/deletions (which are sometimes associated with NHEJ). Nevertheless, inflammation-induced HR events are expected to arise contemporaneously with other classes of mutations. Specifically, both point mutations and HR events arise primarily as a consequence of DNA damage that is present during DNA replication. Thus, HR may serve as an indicator of a more general increase in mutagenesis. Indeed, an association between point mutations and HR events is consistent with observations showing that exposure-induced HR is an excellent predictor of carcinogenicity, which generally arises as the result of multiple classes of mutations [100].

RONS-induced DSBs are rarely caused by direct reaction with the DNA [18,19], but instead are the result of enzymatic processing. Specifically, base excision repair of RONS-induced lesions is associated with gaps that form as repair intermediates [60]. These single strand breaks can become DSBs when repair patches are closely opposed [60,101]. Additionally, replication forks that encounter RONS-induced single strand breaks can break down [21], creating a double strand break. We observed an increase in DSBs under both the conditions of isolated bouts of inflammation, and overlapping bouts of inflammation. Interestingly, under conditions where proliferation from the first bout of inflammation overlaps with acute inflammation from the second bout of inflammation, we observed that DSBs were greatly increased compared to conditions without overlap. This observation is consistent with the possibility that DSBs form in a replication-dependent manner as a result of replication fork breakdown.

In the FYDR direct repeat substrate, full-length *Eyfp* sequence can be reconstituted by several HR mechanisms. For example, if there is a fork breakdown event during DNA replication, misinsertion of the double-strand end can restore full length *Eyfp*, leading to a gain of one repeat unit (a rearrangement at the FYDR substrate, Fig. 1A). Importantly, the FYDR substrate is similar in size to *Alu* repeats (~500 bp vs ~300 bp), which make up almost 10% of the human genome and are frequent sites of HR-induced rearrangement formation [102]. HR between *Alu* repeats can yield deletions, duplications and translocations [102]. *Alu*-mediated rearrangements have been shown to activate oncogenes in cancer [103] and to inactivate tumor suppressor genes such as p53 [104]. Further, HR-driven rearrangements between *Alu* repeats have been shown to drive carcinogenesis in inflammation-associated cancers [36, 105]. Thus, HR events that occur in FYDR mice after replication fork repair are related to genetic changes that are relevant for carcinogenesis in humans.

Alkylating agents are abundant in our environment, endogenously produced in our cells, and used at high doses as cancer therapeutics. Understanding factors that modulate alkylation-induced mutations is therefore relevant both to cancer etiology and to cancer recurrence. We show here that inflammation-induced cell proliferation acts synergistically with alkylation damage to induce sequence rearrangements (Fig. 9). Thus, one potential factor when considering the underlying mechanisms by which chronic inflammation promotes cancer is that the inflammatory response sensitizes tissue to exposure to DNA damaging agents that are in our

environment and in our food. Furthermore, as proliferation itself is sufficient to increase susceptibility to DNA damage induced sequence rearrangements [106], careful consideration should be given to babies *in utero* and young children for whom high levels of cell proliferation are anticipated to greatly sensitize cells to exposure-induced mutations. Thus, when screening for potentially carcinogenic exposures, it will be important to consider the importance of a person's physiological state when assessing risk, with regard to both chronic inflammatory conditions and stage in development.

Recurrence is the single biggest hurdle in cancer treatment, and mutations are critical in eliciting phenotypic changes that initiate new secondary cancers, promote existing cancer cells, and potentiate drug resistance [1,12–15]. It has recently been demonstrated that mutation rate directly impacts the emergence of drug resistance [14]. While in some cases cancer cells are hypermutable [13], many transformed cells have a normal mutation rate [12], making exposure-induced mutations highly relevant. Tumors generally exist in a chronic pro-inflammatory environment. Associated increases in proliferation of both tumor and stromal cells are anticipated to increase susceptibility to RONS-induced and chemotherapy-induced HR events that can promote metastasis and recurrence (Fig. 9). Novel approaches for treating cancer are currently in development, including staged release of drugs from nanoparticles that increase cell killing by chemotherapeutic agents [107]. These approaches could help minimize treatment-induced mutations and thus slow the emergence of drug resistant or more aggressive cancers.

The observation that there is synergy between conditions that induce hyperproliferation and conditions that cause DNA damage is relevant to millions of people who suffer from chronic inflammation and are thus at increased risk of mutations that drive cancer. In addition, the observation that inflammation sensitizes tissue to alkylation-induced HR is relevant to other exposures that create DNA lesions that inhibit replication, including constituents of food, cigarette smoke, and environmental carcinogens (*e.g.*, aflatoxin, BaP, PhIP). Importantly, although the focus of this work is on HR at an integrated reporter, the FYDR model serves as a powerful tool to learn about more general increases in HR throughout the genome, with their accompanied increased risk of LOH, insertions, deletions, and point mutations, all of which drive cancer (Fig. 9). Through these studies of the dynamic physiological changes associated with inflammation, this work contributes to our fundamental understanding of how inflammation drives genetic changes that cause cancer and calls attention to new avenues to disease prevention and treatment.

Materials and Methods

Ethics statement

All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals, and were approved by the MIT Committee on Animal Care.

Chemicals

Cerulein, methylnitrosourea (MNU), BrdU, soybean trypsin inhibitor and collagenase were purchased from Sigma-Aldrich.

Animals

Female C57Bl/6 p^{un} FYDR mice ([7], 5 to 7 weeks old) were used for measuring HR. Inflammation, proliferation and double-strand breaks were measured using female C57Bl/6 (Taconic) and C57Bl/6 p^{un} FYDR mice (5 to 7 weeks old). Metaplastic and preneoplastic lesions were assayed using male wild type or K-Ras mutant mice (gifts from T. Jacks, MIT) on the FVB

background (8 months old at analysis). Mice were housed in an AAALAC approved, specific pathogen free facility under a 12h light/dark cycle and were fed a standard rodent diet (LabDiet RMH 3000, Purina LabDiet) and autoclaved water *ad libitum*. For measuring HR, litters were split between experimental groups.

Repeated acute pancreatitis

Mice were subjected to 3 episodes of acute pancreatitis on experimental days 0, 4 and 9, or on days 0, 14 and 28. Each episode was elicited by 6 hourly intraperitoneal injections of cerulein (dissolved in PBS, 50 µg/kg for each injection). Control animals did not receive injections, as serial injections of PBS have no effect on HR (S6 Fig.). To assess inflammation, Ki-67 expression, and double-strand breaks, mice were humanely euthanized 12 hours after the first cerulein injection and pancreata were harvested for histological analysis. To assess regenerative cell proliferation by BrdU labeling, mice were dosed with BrdU (75 mg/kg) five days after the first bout of acute pancreatitis. Four hours after BrdU injection, mice were humanely euthanized and their pancreata were harvested and processed for BrdU detection by flow cytometry. To assess homologous recombination, mice were humanely euthanized 10 to 15 days after the last pancreatitis episode, and pancreata were harvested for the FYDR assay.

Chronic pancreatitis

Chronic pancreatic inflammation was elicited by cerulein injections (5 µg dissolved in saline, single intraperitoneal injection, 5 days a week) for 6 months, as described in [108]. Control mice received saline injections. Mice were 2 months old at the beginning of treatment. At 8 months of age, mice were humanely euthanized and pancreata were harvested for histological analysis.

Regenerative proliferation and exogenous DNA damage

Mice received 6 hourly intraperitoneal injections of cerulein (dissolved in PBS, 50 µg/kg for each injection) to induce acute pancreatitis. Control mice received 6 hourly injections of PBS. To assess regenerative proliferation by Ki-67 expression, mice were humanely euthanized five days after acute pancreatitis induction and their pancreata were harvested for histological analysis. To induce exogenous DNA damage during regenerative proliferation, mice were dosed with methylnitrosourea (25 mg/kg, dissolved in PBS, pH 4) five days after cerulein treatment. (Note that the timing in this experiment is different from the timing in the repeated inflammation experiment, as MNU generates DNA damage directly and much faster than inflammation induced by cerulein.) Control mice were dosed with PBS, pH 4. Mice were humanely euthanized 3 to 4 weeks after methylnitrosourea injection and pancreata were harvested for the FYDR assay.

BrdU labeling

Pancreata were disaggregated by mechanical chopping and collagenase V digestion at 37°C for 40 min, followed by gentle pipetting. Cells were collected by centrifugation and were stained with the APC Cell Proliferation Detection Kit (BD Pharmingen) according to the manufacturer's instructions. Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest Pro software. On average, 20 000 cells were analyzed per sample.

Ki-67 immunohistochemistry

Pancreata were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4 μm . After deparaffinization, heat-induced antigen retrieval was performed using a modified citrate buffer (Dako). Ki-67 antibody (rat anti-mouse Ki-67, Dako) was used at a dilution of 1/100 at room temperature for 1 hour. Secondary antibody (biotinylated rabbit anti-rat Ig, Dako) was used at a dilution of 1/100 at room temperature for 20 minutes, and detected using streptavidin-conjugated peroxidase and DAB. Sections were counter-stained with hematoxylin. In repeated inflammation experiments, the percentage of Ki-67 positive nuclei was determined in 20 randomly selected images ($\times 20$) using image analysis software (Visiopharm, Hørsholm, Denmark). In the acute inflammation + MNU experiment, the number of Ki-67 positive nuclei was counted in 15 randomly selected image fields ($\times 20$) in a blinded fashion.

γH2AX immunofluorescence

Sections (4 μm) of formalin-fixed, paraffin-embedded tissue were deparaffinized and antigen-retrieved using modified citrate buffer (Dako). Sections were incubated with primary γH2AX antibody (Millipore) at a dilution of 1/100 at 4°C for 3 hours. Secondary antibody (Alexa Fluor 488 Goat Anti-Mouse IgG, Invitrogen) was used at a dilution of 1/500 at room temperature for 1 hour. Sections were counter-stained with DAPI before imaging. For each section, images of 20 randomly selected image fields were acquired at a magnification of $\times 40$ using ImagePro Plus software (Media Cybernetics). DAPI-stained nuclei were counted using ImagePro Plus, and nuclei containing more than 5 γH2AX foci were counted manually in a blinded fashion.

Homologous recombination assay

***In situ* fluorescent imaging.** Pancreata were immersed in ice cold soybean trypsin inhibitor solution (0.01% in PBS) immediately after harvesting. Pancreata were pressed between glass coverslips separated by 0.5 mm spacers and imaged on a Nikon 80i epifluorescence microscope (Nikon) with a CCD camera (CoolSNAP EZ, Photometrics) using a $\times 1$ objective at a fixed exposure time (2 s). EYFP was detected in the FITC channel. Multipoint images captured using an automated stage (ProScan II, Prior Scientific) and NIS Elements software (Nikon) were stitched automatically or manually in Adobe Photoshop (Adobe Systems). Brightness and contrast were adjusted identically across images, and foci were manually counted in a blinded fashion. Areas of pancreata were determined using ImageJ software (NIH) by manually tracing the pancreas outline.

Flow cytometry. Following imaging, pancreata were disaggregated into single-cell suspensions as described in [7], with minor modifications. Briefly, pancreata were minced with scalpel blades, followed by digestion with collagenase V (2 mg/ml in Hanks' Balanced Salt Solution) for 40 min at 37°C. The resulting suspension was gently triturated to increase mechanical separation and filtered through a 70 μm cell strainer (BD Falcon) into an equal volume of media (DMEM F12 HAM with 20% FBS). Cells were collected by centrifugation, resuspended in 350 μl OptiMEM (Invitrogen) and filtered through 35 μm filter caps into flow cytometry tubes (Beckton Dickinson). Samples were analyzed on a FACScan cytometer (Beckton Dickinson) using CellQuest Pro software (Beckton Dickinson). On average, 1 800 000 cells were analyzed per sample.

Pathological analysis

Pancreata were fixed in 10% buffered formalin, embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin. Pancreata were then examined and scored by a trained

veterinary pathologist in a blinded fashion on a scale of 0 to 4 for the following individual features: inflammation, edema, hemorrhage, acinar degeneration/necrosis, acinar loss/atrophy, fat infiltration, fibrosis, acinar to ductal metaplasia (ADM), acinar/ductal hyperplasia, acinar dysplasia/neoplasia and ductal dysplasia/hyperplasia. For the acute studies, only a few relevant subsets were analyzed and scored, whereas for the chronic studies, the full set of criteria was assessed.

Statistics

Inflammation, proliferation and double-strand break indices were compared with Student's *t*-test. Numbers of recombinant foci, recombinant cell frequencies, and pathological scores do not follow a normal distribution and were compared with the Mann–Whitney *U*-test. All statistical analyses were performed in GraphPad Prism, Version 5.02 (GraphPad Software). A *P* value less than 0.05 was considered statistically significant.

Supporting Information

S1 Fig. HR at the FYDR recombination substrate is detected by fluorescence after gene conversion, sister chromatid exchange, and replication fork repair. Each expression cassette is missing different essential *EYFP* coding sequences, such that neither is able to express functional protein. Gene conversion can lead to the transfer of sequence information from one cassette to the other, restoring full-length *EYFP* coding sequence and giving rise to fluorescence. Each cassette can be the donor or the recipient in a gene conversion event. The entire HR reporter is copied during S phase, making it possible for crossovers between sister chromatids (gene conversion with crossover) to reconstitute full-length *EYFP*. Note that a long tract gene conversion event would be indistinguishable. HR repair of a broken replication fork can also be detected using the FYDR substrate. The breakdown of a replication fork moving from left to right is shown. Reinsertion of the broken $\Delta 3egfp$ end into the $\Delta 5egfp$ cassette can restore full length *EYFP*. *EYFP* can analogously be restored by repair of forks moving in the opposite direction (not shown). Single strand annealing initiated by a DSB between the repeated cassettes can be readily repaired, but these events will not reconstitute full-length EGFP and thus SSA cannot be detected. (TIF)

S2 Fig. Chronic cerulein treatment leads to dysplastic and preneoplastic changes in K-Ras mice. (A) Pancreas from mock treated K-Ras mutant mouse. Inflammation, acinar atrophy and interstitial fibrosis (arrow) are detectable. Acinar-to-ductal metaplasia is sparse. H&E staining. Original magnification, $\times 100$. Scale bar = 160 μm . (B) Pancreas from K-Ras mutant mouse treated with chronic cerulein. Small focal proliferation of acinar tubules (thick arrow) with architectural and cytological atypia (dysplasia, low grade) surrounded by inflammation. Few acini with mucous metaplastic changes (thin arrow) are also present. Original magnification, $\times 400$. Scale bar = 40 μm . (C) Histological scores for acinar-to-ductal metaplasia in mock and chronic cerulein treated K-Ras mutant mice. Detailed scoring criteria are described in *Methods*. Each symbol denotes data from one mouse. ***, $P < 0.001$, Mann–Whitney *U*-test. (D) Histological scores for dysplasia/neoplasia in mock and chronic cerulein treated K-Ras mutant mice. Detailed scoring criteria are described in *Methods*. Each symbol denotes data from one mouse. **, $P < 0.01$ (Mann–Whitney *U*-test). (TIF)

S3 Fig. Independent and overlapping bouts of pancreatic inflammation. (A) For independent bouts of inflammation, three acute cerulein pancreatitis events were induced two weeks apart, and inflammation and proliferation were assessed at the second (analysis time A) and third (analysis time C) bout of inflammation. HR was quantified 10 to 15 days after the last

pancreatitis event. (B) For overlapping bouts of inflammation, three acute cerulein pancreatitis events were induced on days 1, 4 and 9. Inflammation and proliferation were assessed at the second (analysis time B) and third (analysis time D) bout of inflammation. HR was quantified 10 to 15 days after the last pancreatitis event. (C) Pancreas section from a control mouse shows healthy tissue. (D,E) Treatment with cerulein (both independent and overlapping) results in edema and an inflammatory infiltrate chiefly of neutrophils, indicating acute inflammation. (F) Ki-67 immunohistochemistry shows low levels of baseline proliferation in control pancreata. (G) Cell proliferation remains low in the pancreas during acute inflammation. (H) During regeneration from acute inflammation, Ki-67 positive nuclei appear, indicating regenerative proliferation. (I) Immunohistochemical detection of γ H2AX phosphorylation in pancreas sections show low levels of DSBs in healthy pancreata. (J) During independent bouts of inflammation, nuclei with γ H2AX foci (arrowhead) become apparent. (K) During overlapping bouts of inflammation, γ H2AX positive nuclei are visible. (C-E) Original magnification, $\times 10$. Scale bar = 200 μ m. (F-H) Original magnification, $\times 20$. Scale bar = 100 μ m. (I-K) Original magnification, $\times 40$. (TIF)

S4 Fig. Inflammation, proliferation and DSBs in independent and overlapping bouts of inflammation. Inflammation, cell proliferation and γ H2AX foci formation were quantified in pancreas sections from mice treated with independent bouts of inflammation (blue bars) and with overlapping bouts of inflammation (purple bars). (A,B) Cerulein induces inflammation in both independent ($n = 7$) and overlapping ($n = 8$) treatment regimens. Severity of inflammation in control and cerulein-treated mice was quantified by a trained pathologist. (C, D) Quantification of nuclei positive for the proliferation marker Ki-67 shows no increase in independent bouts of inflammation ($n = 7$), and a large increase in overlapping bouts of inflammation ($n = 8$). (E,F) Quantification of nuclei positive for the DSB marker γ H2AX (nuclei with >5 foci) shows a moderate increase in independent bouts of inflammation ($n = 3$), and no significant increase in overlapping bouts of inflammation ($n = 3$). Data are mean \pm SEM. See [Methods](#) for detailed pathological scoring criteria. Statistical testing could not be performed in groups containing only zero values. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ (Student's t -test). (TIF)

S5 Fig. Low-dose MNU treatment does not induce HR. Animals received MNU (7.5 mg/kg) in a single intraperitoneal injection, and HR was evaluated 3 to 5 weeks later. There is no significant difference between the numbers of fluorescent foci in control ($n = 15$) and MNU-treated ($n = 14$) mice. Symbols represent data from individual mice, horizontal bars show medians. ns, not statistically significant (Mann-Whitney U -test). (TIFF)

S6 Fig. Repeated intraperitoneal PBS injections have no effect on HR in the pancreas. Mice received single (*Left*, $n = 85$) or multiple (*Right*, $n = 22$) intraperitoneal PBS injections and the numbers of fluorescent foci in their pancreata were determined after *in situ* imaging as described in *Methods*. Symbols represent data from individual mice, horizontal bars show medians. ns, not statistically significant (Mann-Whitney U -test). (TIF)

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Author Contributions

Conceived and designed the experiments: OK BPE. Performed the experiments: OK GG WO. Analyzed the data: OK WO SM BPE. Wrote the paper: OK BPE.

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Exhibit 106

REVIEW

Possible Role of Ovarian Epithelial Inflammation in Ovarian Cancer

Roberta B. Ness, Carrie Cottreau

Ovarian cancer is a commonly fatal disease for which prevention strategies have been limited, in part because of a lack of understanding of the underlying biology. This paper reviews the epidemiologic literature in the English language on risk factors and protective factors for ovarian cancer and proposes a novel hypothesis that a common mechanism underlying this disease is inflammation. Previous hypotheses about the causes of ovarian cancer have attributed risk to an excess number of lifetime ovulations or to elevations in steroid hormones. Inflammation may underlie ovulatory events because an inflammatory reaction is induced during the process of ovulation. Additional risk factors for ovarian cancer, including asbestos and talc exposure, endometriosis (i.e., ectopic implantation of uterine lining tissue), and pelvic inflammatory disease, cannot be directly linked to ovulation or to hormones but do cause local pelvic inflammation. On the other hand, tubal ligation and hysterectomy act as protective factors, perhaps by diminishing the likelihood that the ovarian epithelium will be exposed to environmental initiators of inflammation. Inflammation entails cell damage, oxidative stress, and elevations of cytokines and prostaglandins, all of which may be mutagenic. The possibility that inflammation is a pathophysiologic contributor to the development of ovarian cancer suggests a directed approach to future research [J Natl Cancer Inst 1999;91:1459–67]

Ovarian cancer is the gynecologic cancer most likely to result in death among women (1), yet the pathophysiology underlying epithelial ovarian cancer is not clearly established. For many years, two dominant hypotheses—the ovulation hypothesis (2–4), which relates ovarian cancer risk to incessant ovulation, and the pituitary gonadotropin hypothesis (5), which implicates elevations in gonadotropin levels acting in concert with estrogen—have sought to explain the genesis of this disease. Epidemiologic and biologic data have not been entirely consistent with either of these hypotheses. At the same time, a growing body of epidemiologic evidence suggests that factors causing epithelial inflammation are involved in ovarian carcinogenesis. Such factors include asbestos and talc exposures, endometriosis, and pelvic inflammatory disease (PID). Conversely, there appear to be protective effects of tubal ligation and hysterectomy, which may reduce the exposure from local genital tract irritants. We first briefly review evidence for and against the ovulation and gonadotropin hypotheses. We then propose that inflammation may work in conjunction with, and in addition to, ovulation and steroid hormones in mediating epithelial ovarian cancer risk (Fig. 1).

In this review, only epithelial ovarian cancers will be discussed because they account for about 90% of all ovarian cancers. We will not discriminate between invasive and noninvasive

tumors, since both have similar risk factors. Also, we acknowledge the potential heterogeneity between mucinous and other epithelial ovarian tumor types (6,7), but histology-specific considerations are beyond the scope of this review.

Studies were identified for this review by searching the English language literature in the MEDLINE® database and by an extensive review of bibliographies from articles found through that search.

EVIDENCE SUPPORTING THE PITUITARY GONADOTROPIN AND OVULATION HYPOTHESES

The factors that afford the greatest overall risk reduction for ovarian cancer in female populations are parity (number of live births) (6,8–36), oral contraceptive use (6,8–16,24,31,32,35–45), and prolonged breast-feeding (31,46). During pregnancy, very high levels of estrogen and progesterone suppress levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and disallow ovulation; during oral contraceptive use, stable levels of estrogens and progestins inhibit the gonadotropins and their ability to stimulate ovulation; and during breast-feeding, low levels of estrogen and LH suppress ovulation (47). That these reproductive and contraceptive factors are protective suggests a common effect through ovulation or steroid hormones. Oral contraceptive use, parity, and breast-feeding each provide a reduction in risk for two to three decades after their cessation, so that they must trigger biologic events that do not clinically manifest themselves as cancer until many years thereafter (48).

If fertility drugs were found to influence the development of ovarian cancer, this influence would also potentially support both the ovulation and gonadotropin hypotheses, since these drugs both elevate gonadotropin levels and cause superovulation. However, the literature (49,50) is conflicting regarding the association between the use of fertility drugs and ovarian cancer.

SCRUTINIZING THE PITUITARY GONADOTROPIN HYPOTHESIS

The pituitary gonadotropin hypothesis suggests that critical events in the transformation to ovarian cancer are the entrapment

Affiliations of authors: R. B. Ness, Department of Epidemiology, University of Pittsburgh Graduate School of Public Health, University of Pittsburgh Cancer Institute, and Magee-Womens Research Institute, Pittsburgh, PA; C. Cottreau, Department of Epidemiology, University of Pittsburgh Graduate School of Public Health.

Correspondence to: Roberta B. Ness, M.D., M.P.H., University of Pittsburgh Graduate School of Public Health, 130 DeSoto St., Rm. 513, Parran Hall, Pittsburgh, PA 15261 (e-mail: repro@vms.cis.pitt.edu).

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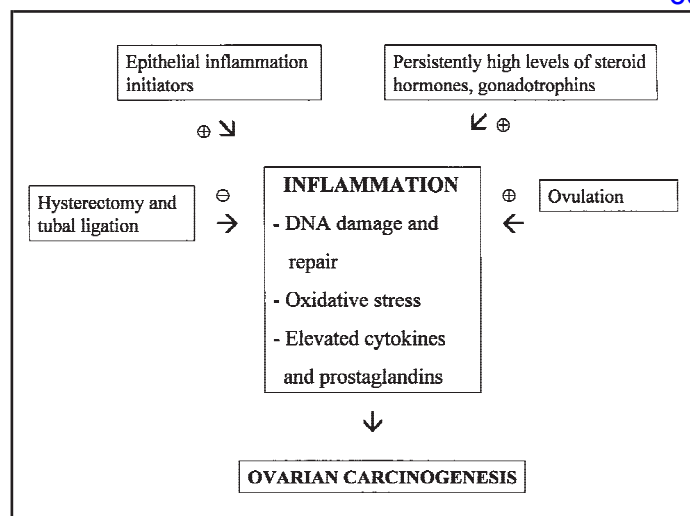


Fig. 1. Inflammation as a common mechanism underlying ovarian cancer.

of surface epithelium in inclusion cysts followed by stimulation of the entrapped epithelium by estrogen or estrogen precursors, particularly in the presence of high and persistent levels of gonadotropins (LH and FSH) (5). Several observations do not completely fit the pituitary gonadotropin hypothesis. High estrogen levels alone could not be the whole story behind mutagenicity because estrogen levels are at their highest during pregnancy, a reproductive event that is strongly protective for ovarian cancer (48). In addition, one study (51) found no estrogen receptors in epithelium on the surface of the ovary or in inclusion cysts. Cramer and Welch (5) illustrated the nature of the proposed interplay between gonadotropins and estrogens and suggested that disruption of negative feedback to the pituitary in the presence of an otherwise normal ovarian steroidal environment (e.g., by transplanting the ovary to the spleen wherein ovarian hormones would be degraded by the liver) would elevate gonadotropins and stimulate ovarian mutagenesis. A pharmacologic equivalent to this would be use of medications, such as barbiturates, halogenated hydrocarbon pesticides, anti-inflammatory medications, and antihistamines, that would degrade estrogen at a greater than normal rate. However, to our knowledge, there has not been any evidence that such medications increase the risk of ovarian cancer (52). These authors (5,53) also proposed that premature ovarian failure or early menopause could be associated with elevated ovarian cancer risk via high gonadotropin levels. However, there is little evidence that age at natural menopause influences risk (32,46).

Furthermore, in the only prospective study to examine this question directly (54), gonadotropin levels measured from serum stored many years prior to outcome were not associated with the occurrence of ovarian cancer. Helzlsouer et al. (54) analyzed levels of LH, FSH, and other hormones among case patients with ovarian cancer and control subjects arising from a prospective population-based serum bank study. Of 20 305 participants from whom serum had been collected and frozen, 31 who were not taking hormone replacement therapy (HRT) at baseline developed ovarian cancer a mean of 8 years after blood collection. These case patients were matched to 62 control subjects on age, menopausal status, and, for premenopausal women, number of days from the beginning of the last menstrual period. Mean levels of FSH, LH, and estrogens were somewhat lower among

case patients with ovarian cancer than among control subjects, whereas the androgens androstenedione, dihydroepiandrosterone, and dihydroepiandrosterone sulfate were associated with an increased risk. These results do not support the hypothesis that elevated pituitary gonadotropin levels increase ovarian cancer risk. However, limitations of the study were the measurement of hormones at a single point in time, the inclusion of premenopausal women without precise determination of timing of blood collection within the menstrual cycle, the small number of cases of ovarian cancer, and the limited adjustment for confounding factors.

A more complex issue that is somewhat difficult to reconcile with the gonadotropin hypothesis is that postmenopausal estrogen use has been modestly, albeit inconsistently, associated with increased risk for ovarian cancer (7,8,11,12,14,18,24,36,55–62). A recent meta-analysis (63), including 11 articles with data from 21 studies, did show a small increase in overall risk with HRT use (relative risk [RR] = 1.15; 95% confidence interval [CI] = 1.05–1.27) with a somewhat higher risk, albeit of borderline significance, among users for more than 10 years' duration (RR = 1.27; 95% CI = 1.00–1.61). Rodriguez et al. (62) in the prospective Nurses Health Study found 18 cases in 5000 person-years among long-term users (>11 years), for an RR of 1.7 (95% CI = 1.1–2.8). Postmenopausal estrogens reduce gonadotropins and increase estrogen levels. To the degree that the gonadotropin hypothesis predicted that excess LH and FSH stimulate mutagenesis, these findings would seem to counter the predictions of the hypothesis. However, if the hormonal mechanism more relevant to the thesis of the gonadotropin hypothesis were that of estrogen elevation, then these findings would indeed fit the data. Taken together, the literature reviewed above does not fully support the gonadotropin hypothesis, although it is quite possible that steroid hormones do play some role in pathogenesis.

SCRUTINIZING THE OVULATION HYPOTHESIS

The ovulation hypothesis states that excessive ovulation damages the ovarian epithelium, from which epithelial ovarian cancer arises (2). This hypothesis proposes that repeated cell damage translates into an enhanced potential for aberrant DNA repair, inactivation of tumor-suppressor genes, and subsequent mutagenesis (3,4). Perhaps the most complex issue to reconcile with the ovulation hypothesis is whether ovulatory infertility increases the risk for ovarian cancer. Ovulatory infertility is the result of a lack of ovulation and so should not elevate the risk of ovarian cancer according to this hypothesis. Although several studies [reviewed at length elsewhere (49)] have shown that ovarian cancer is associated with difficulty in achieving pregnancy (8,21,31,64–69), there has been inconsistency regarding the type of infertility associated with risk. With regard to ovulatory infertility, Rossing et al. (64) examined records of women who presented to infertility clinics in Seattle, WA, during the period from 1974 through 1985 and who were subsequently identified through cancer registry information if they developed ovarian cancer. Based on small numbers, the RR for ovulatory abnormalities was 3.7 (95% CI = 1.4–8.1) when compared with population-based expected rates of ovarian cancer. This analysis was limited by the likelihood that the external comparison population would likely be more parous, more likely to have used oral contraceptives, and therefore at a lower ovarian cancer risk—hence, resulting in an inflation of the observed RR. In fact, when Rossing et al. compared women with ovulatory infertility with

internal control subjects who had other infertility diagnoses, the risk of ovarian cancer was 1.8 (95% CI = 0.5–6.1). Venn et al. (65) published data from a larger retrospective cohort study of women attending an *in vitro* fertilization clinic and compared their rates with population-based ovarian cancer rates. Again, infertility was associated with ovarian cancer, but only for women with unexplained infertility (odds ratio [OR] = 19.2; 95% CI = 2.2–165) and not for women with ovulatory infertility. In summary, because anovulation is only one among several possible causes of infertility, this limited literature neither supports nor refutes the ovulation hypothesis.

Factors that reduce ovulation do not proportionally reduce the risk of ovarian cancer (24,46). First proposed by Risch et al. (24) and later demonstrated by Whittemore et al. (46), 1 year of delayed menarche or of early menopause was associated with a much less marked reduction in ovarian cancer risk than was 1 year of term pregnancy, 1 year of breast-feeding, or 1 year of oral contraceptive use. Were the ovulation hypothesis to hold, there is no reason to imagine that various sources of ovulation cessation would differentially impact risk. However, age at menarche and age at menopause may less accurately reflect ovulatory function than does pregnancy or oral contraceptive use; the initiation and cessation of menses do not reflect the initiation and cessation of ovulation (70). Nevertheless, suppression of ovulation cannot fully account for the risk reductions observed in epidemiologic studies. Assuming that ovulations occur over a period of at least 20 years, a full-term pregnancy would be expected to reduce ovarian cancer risk by 5%, whereas Whittemore et al. (46) observed about a 15% reduction in risk for each pregnancy after the first.

EPIDEMIOLOGIC DATA SUPPORTING THE ROLE OF LOCAL INFLAMMATION IN OVARIAN CANCER RISK

Several types of exposure that do not directly affect ovulation or steroid hormone levels but that do enhance local inflammation have been implicated as ovarian cancer risk factors. Reduced passage of inflammatory toxins from the lower to the upper genital tract may also reduce risk.

TALC AND ASBESTOS EXPOSURE

In the early 1960s, it was recognized that female asbestos workers had an increased risk of developing ovarian cancer and other intra-abdominal neoplasms (71,72). Subsequent retrospective cohort studies of women who were employed in industries wherein they might encounter heavy asbestos exposure (73–75) found about a twofold excess of ovarian cancers over what was expected, with a dose–response relationship suggested. Heller et al. (76) documented that substantial amounts of asbestos fiber could be detected in the ovarian tissues of women whose fathers or husbands worked in occupations in which asbestos exposure was high. The rates of finding asbestos in ovarian tissue were twice as high in women with household exposure as in women without such an exposure history. Animal models (73,77,78) provide some support for the suggestion that asbestos exposure may cause ovarian cancer. Intraperitoneal injection of asbestos into guinea pigs and rabbits results in changes in the ovarian epithelium similar to those seen in early ovarian cancer in women; similar changes were found among 20% of the exposed and 0% of the unexposed animals (77). However, whereas asbestos was cytotoxic to hamster ovary cells *in vitro* (78), it had no effect on the ovaries of mice and hamsters *in vivo* (77).

Although household-related asbestos exposure may be related to dust on the clothing, with those who launder the clothing at increased risk of cancer, it is also possible that exposure occurs through sexual intercourse with particles traveling from the lower to the upper genital tract. Traffic of endogenous cells and pathogens from the lower to the upper genital tract has been shown to be common (79). This fact links cervicitis, i.e., sexually transmitted infection of the lower genital tract epithelium, to PID. It may also link asbestos exposure and talc use to ovarian epithelial inflammation.

Talc, which is structurally similar to asbestos, has repeatedly been related to ovarian cancer. Prior to 1976, talc was commonly contaminated with asbestos, so that the early studies relating talc to ovarian cancer may have been confounded by the asbestos–ovarian cancer relationship (80). More recent findings are less likely to be solely driven by the asbestos relationship.

At least 12 epidemiologic studies (8,81–91) have evaluated the use of talc in relationship to ovarian cancer. Eight of these studies (81–87,90) reported an elevated cancer risk among women whose powder exposure was described as a “dusting of the perineum,” with ORs ranging from 1.3 to 3.9. Two other studies (8,88) found a very small elevation in risk with the use of a more general exposure definition, and one study (89) found no association. In the most extensive and focused analysis to date, Cook et al. (81) interviewed 313 case patients with ovarian cancer and 422 control subjects regarding exposure to a variety of powder products used in a series of ways (e.g., perineal dusting, diaphragm storage, powder on sanitary napkins, and genital deodorant spray). Both talc-containing and non-talc-containing baby or bath powder products were associated with an elevated risk of ovarian cancer; each way of using it, with the exception of diaphragm storage, was also associated with an elevated risk of ovarian cancer. A limited number of studies (8,81,90,92) have examined the potential for a dose–response relationship. Some studies have shown some increase in risk with more frequent exposure (83,86), longer exposure (86), and greater total number of lifetime applications (86). However, other studies (8,81,90) have not shown any dose–response relationship. The link between talc exposure and ovarian cancer is limited by a lack of supportive animal data and an inconsistency in the detection of talc in the ovarian tissue of women who reported heavy use (91). Nevertheless, the consistency of an association between talc use and ovarian cancer in a series of well-conducted studies of varying design suggests that talc use may represent another environmental exposure that enhances epithelial inflammation and thereby either initiates or promotes ovarian carcinogenesis.

ENDOMETRIOSIS

Endometriosis is the presence of endometrial tissue outside the lining of the uterus. Although the cause of endometriosis is unknown, it is clear that the implantation of ectopic endometrial tissue is associated with a local inflammatory reaction, including macrophage activation, and elevation of cytokines and growth factors.

Ovarian tumors arise out of ovarian endometriosis in 0.3%–0.8% of case patients who are followed clinically (93,94). In the most extensive epidemiologic study to date, Brinton et al. (95) assessed cancer outcomes among 20 686 women with endometriosis who were hospitalized in Sweden. Hospitalizations were identified through the nationwide Swedish Inpatient Registrar, and outcomes were identified through the National Swedish

Cancer Registry after a mean of 11.4 years of follow-up. The risk of ovarian cancer was elevated 2.5-fold for women followed for 10 or more years, and the risk rose to more than fourfold among women whose endometriosis was located in the ovaries. Unfortunately, this study did not control for parity or oral contraceptive use, which might have led to an inflated estimate of risk. However, there is also substantial clinical support for an association between endometriosis and ovarian cancer.

Several case series (93,96–101) have demonstrated cancer tumorigenesis that arises from endometriosis. Sampson (102), who documented the first case, outlined a set of criteria for establishing the existence of such a cancerous transformation. These criteria include the following: 1) demonstration of both cancerous and benign endometrial tissues in the same ovary, 2) demonstration of cancer arising in the tissue and not invading from another source, and 3) demonstration of a histologic relationship between invasive and benign components. Reviewing the literature, Heaps et al. (93) noted that 165 cases have been published that meet these criteria. Almost 80% of these malignant transformations arose from ovarian endometriosis, and the rest came from extragonadal sites. Endometrioid adenocarcinomas accounted for 69% of lesions, followed by clear-cell carcinomas (13.5%) and sarcomas (11.6%). This is a far higher proportion of endometrioid and clear-cell tumors than is found among ovarian cancers in general (10%–20% and 3%–10%, respectively), which again points to a possible transformation from endometriosis to specific types of endometrial cancer (103). One case report (99) documented the experience of a woman who, on biopsy, first showed atypia within ovarian endometriosis and then 3 years later had a clear-cell ovarian carcinoma arising from the same ovary. Finally, Sainz de la Cuesta et al. (96) found endometriosis among about 40% of women with stage I endometrioid or clear-cell ovarian carcinoma, about one third of which were carcinomas arising out of endometriosis. Czernobilsky and Morris (104) also showed that mild cytologic atypia occurred in about 20% of endometriosis lesions and that severe atypia, a probable precursor of ovarian cancer, occurred in 3.6%. Taken as a whole, these data strongly support a temporal pattern of transition from simple endometriosis to atypical endometriosis to ovarian cancer.

HYSTERECTOMY AND BILATERAL TUBAL LIGATION

Hysterectomy without oophorectomy and tubal ligation both have been associated with reductions in the risk for ovarian cancer (105–115). ORs have ranged from 0.03 to 0.8 for hysterectomy and from 0.2 to 0.9 for tubal ligation. Some authors (105–107) found that the protective effect for hysterectomy waned after 5–20 years and suggested that the observed protection afforded by these procedures might result from screening whereby ovaries examined at the time of surgery and found to be abnormal were removed. However, other authors (6,108,114) found that the protection afforded by hysterectomy or tubal ligation continues for 20–25 years after the procedure. Green et al. (114) proposed that the mechanism whereby hysterectomy and tubal ligation protect against ovarian cancer is by cutting off the pathway between the lower and the upper parts of the genital tract, thereby disallowing proinflammatory exposures from reaching the ovarian epithelium. This may account for the finding by Whittemore et al. (106), who reported no protective effect of hysterectomy in women who had a prior bilateral tubal ligation but found a reduction in risk for women with no prior tubal

ligation. Furthermore, Whittemore et al. showed that tubal ligation protected against the effect of talc. Women who used talc but had never had surgical sterilization were at 30% increased risk of cancer, whereas women who used talc but had a tubal ligation had a 50% reduction in risk. Thus, talc exposure may occur via ascension of particles from the lower to the upper part of the genital tract and tubal ligation severs this route of ovarian exposure. However, the risk reduction associated with tubal ligation or hysterectomy may be larger than would be expected, presuming that these procedures protect the ovarian epithelium from exposure to known inflammants, particularly because only a subset of women is exposed to talc or asbestos. The probable explanation for the fact that risk reduction for tubal ligation hysterectomy is larger than expected lies in the role of as yet unidentified environmental exposures. For example, sexually transmitted pathogens may act via inflammation to increase risk (*see below*). The inflammation hypothesis challenges investigators to search for other exposures that may gain access to the upper genital tract through the lower genital tract and initiate an inflammatory response.

PELVIC INFLAMMATORY DISEASE

PID is a condition consisting of inflammation of the endometrium, tubes, and ovaries as a result of sexually transmitted infections that ascend from the lower to the upper part of the genital tract. Two case-control studies (34,116) have linked PID with ovarian cancer risk. A third study (117), in which a very small proportion of women (and, therefore, total number of women) reported previous PID, did not. The latter study (117) is likely limited not only by power but also potentially by underreporting of prior PID. Shu et al. (34) first reported a substantial but statistically nonsignificant relationship (OR = 3.0; 95% CI = 0.3–30.2) among a handful of affected case patients and control subjects in Shanghai, China. Risch and Howe (116) subsequently demonstrated the relationship in a study involving 450 case patients with ovarian cancer and 565 control subjects residing in and around Toronto, Canada. They found an increased risk of ovarian cancer among women who had had an episode of PID (OR = 1.5; 95% CI = 1.1–2.1). The relationship between PID and ovarian cancer was most evident in women who had had PID at an early age, were nulliparous, and were infertile. Moreover, there was an increasing trend in risk with increasing number of PID episodes. Each episode of PID promotes a greater and greater inflammatory response, resulting in increasing damage to ovarian and tubal structures and a greater chance of tubal infertility (which, if occurring before the first birth, would manifest itself as nulliparity). Indeed, in the previously mentioned retrospective study of the cohort of infertile women (64), those with tubal infertility were at a threefold increased risk of ovarian cancer. The RR for tubal infertility was of the same order of magnitude as it was for ovulatory infertility, albeit involving a smaller number of individuals and not reaching statistical significance. PID produces infertility by causing inflammation of and damage to the fallopian tube wherein the ovum reaches the uterus, rather than by any effect on ovulation (*see below*). Thus, the finding that PID is associated with ovarian cancer, particularly when there has been resultant chronic inflammation and infertility, is consistent with an inflammatory origin for ovarian cancer.

ANTI-INFLAMMATORY MEDICATIONS

One way to evaluate the role of inflammation in ovarian cancer is to examine the effect of anti-inflammatory medications on risk. Cramer et al. (52) asked 563 case patients with ovarian cancer and 523 population-based control subjects about their lifetime history of anti-inflammatory medication use. The OR for ovarian cancer associated with at least 6 months of once-per-week aspirin use was 0.75 (95% CI = 0.52–1.10) and for ibuprofen use was 1.03 (95% CI = 0.64–1.64). Limitations of this study included the modest number of case patients exposed to long-term aspirin use and the smaller number exposed to ibuprofen, which resulted in broad CIs around ORs; the inclusion of women with modest use of nonsteroidal anti-inflammatory medications as exposed; and the lack of dose or duration data for aspirin or ibuprofen use. Previous studies showing a protective benefit of aspirin use for colon cancer have typically used a more restrictive definition of exposure, such as aspirin use at least two to three times per week, and have more clearly shown an effect for aspirin use than for other nonsteroidal medications, predominantly because only for aspirin have the number of exposed individuals been sufficient to provide stable estimates (118). Indeed, in the only other published study examining the role of analgesics on ovarian cancer risk (89), among 189 women with epithelial ovarian cancers, the adjusted RR for infrequent use was 0.78 (not statistically significant), whereas the adjusted RR for frequent use was 0.51 ($P = .05$). Thus, further investigation of the impact of anti-inflammatory medications on ovarian cancer is warranted.

BIOLOGIC RATIONALE FOR THE ROLE OF INFLAMMATION IN OVARIAN CANCER RISK

Ames et al. (119) argued that carcinogenesis in general may be mediated by oxidative damage to DNA. The general theory was based on the finding that mutations in several critical genes, such as the p53 tumor suppressor gene, can lead to tumors. Damage to the DNA constituting these genes may contribute to mutagenicity, to a degree that depends on the degree of damage, the effectiveness of endogenous repair mechanisms, and the rates of cell division. More rapidly dividing cells would be most prone to errors in DNA replication and repair (120).

Inflammation, by its nature, produces toxic oxidants meant to kill pathogens. These oxidants cause direct damage to DNA, proteins, and lipids and may, therefore, play a direct role in carcinogenesis (121). At the same time, chronic inflammation is associated with increased cell division. Rapid cell division gives rise to the potential for replication errors with resultant DNA repair; aberrant DNA repair, particularly at key regulatory sites (e.g., tumor suppressor DNA regions), may increase the risk for mutagenesis (119). Finally, bioactive substances, such as cytokines, growth factors, and prostaglandins, that are synonymous with inflammation may play an important role in ovarian mutagenesis. Ovarian epithelial cells secrete cytokines, including interleukin 1, interleukin 6, and macrophage colony-stimulating factor, among others (122). Auersperg et al. (123) pointed out that these same factors are also produced by ovarian cancer cells and suggested that the recruitment of normally secreted cytokines into dysregulated autocrine loops may be important in neoplastic progression. Prostaglandins have multiple effects that favor tumorigenesis (124). For example, prostaglandins are more common in ovarian malignant tumors than in normal cells (125),

overexpression of prostaglandins increases the invasiveness of tumor cells, and inhibitors of cyclooxygenase activity (and therefore prostaglandin formation) protect against a variety of cancers in animals (124). Epidemiologic studies have shown that long-term use of nonsteroidal anti-inflammatory medications generally reduces the risk of colon cancer in both men and women (118,126,127) and breast cancer in women (128).

Ovulation may be mutagenic. The process of ovulation requires disruption of the ovarian epithelium (129,130). Degenerative epithelial cells adjacent to the site of follicular rupture are shed from the ovarian surface, presumably through apoptosis (i.e., programmed cell death). The wound that ensues from cell loss and follicular extrusion is repaired by the proliferation of epithelial cells from the perimeter of the ruptured follicle. In the process, inclusion cysts are formed as surface epithelial cells become entrapped in the ovarian wound created during ovulation. There has been speculation that inclusion cysts are among the ovarian surface changes that represent a path of differentiation that is less plastic than the relatively pluripotent normal ovarian epithelium and more likely to proceed to ovarian carcinogenesis (130). This suggestion comes from two observations. First, women with ovarian cancer are more likely to have inclusion cysts in the contralateral ovary (131); however, this finding was not confirmed in another study (132). Second, in an unblinded study (133), ovaries of women at high familial risk of developing ovarian cancer, compared with ovaries of normal women, were more likely to have multiple inclusion cysts as well as papillomatosis, deep invaginations, epithelial pseudostratification, and/or hyperactive stroma. Women with a genetic predisposition to ovarian cancer may thus have ovarian epithelium that is already committed to ovarian carcinogenesis, a feature of which is an excess of inclusion cysts.

There are also data from animal studies and limited human studies to support the hypothesis that ovulation may trigger cellular events that result in carcinogenesis. Hyperovulatory hens have a markedly increased likelihood of developing ovarian adenocarcinomas, as do rats with hyperproliferating ovarian epithelial cells (134,135). In women, mutations of the p53 tumor suppressor gene were associated with an increased number of lifetime ovulations in a study by Schildkraut et al. (120). Mutations of the p53 gene are the most common molecular alterations in ovarian cancer and are thought to result from spontaneous errors of DNA synthesis during cell proliferation (136). Risch (137) questioned the validity of these results on the basis that case patients with p53 mutations were older, had poorer tumor differentiation, and had disease of distant rather than of local or regional stage at diagnosis, perhaps indicating that p53-positive tumors are diagnosed later in the neoplastic process. Schildkraut et al. (138) reanalyzed the data matching on age and then on stage and replicated the original findings. However, a more recent case-control study (139) was unable to confirm the association between lifetime ovulations and p53 mutations.

Mutagenicity induced by ovulation may be mediated by inflammation. Ovulation is associated with a marked inflammatory process at the level of ovulatory follicles (140). Many inflammatory mediators, including vasoactive agents such as bradykinin and inflammatory and anti-inflammatory substances such as prostaglandins and leukotrienes, are locally elevated during ovulation. Epithelium in the neighborhood of inclusion cysts is brought in closer proximity to these substances. Follicle rupture probably involves tissue remodeling, with high cell turn-

over, that is also characteristic of inflammatory reactions. Thus, the process of ovulation is intimately related to inflammation. In particular, epithelium in and around the site of ovulation may replicate more actively, come into contact with cytokines and prostaglandins, and may be subject to oxidative stress, thereby enhancing the risk of mutagenesis.

PREDICTIONS FROM THE INFLAMMATION HYPOTHESIS AND SUGGESTIONS FOR FUTURE RESEARCH

Direct induction of inflammation as a result of endometriosis, talc and asbestos exposure, and PID, as well as ovulation itself, may act to promote ovarian tumorigenesis. There would be several ways to help demonstrate the veracity of this hypothesis. First, anti-inflammatory medications should reduce the occurrence of ovarian cancer. Aspirin use was associated with a reduction in ovarian cancer risk in one previous epidemiologic study; ibuprofen was not (52). Further studies are needed to examine this association. Populations of women with substantial exposures to anti-inflammatory medications, such as those with connective tissue diseases, may be at lower than expected risk, as long as their disease does not inflame the ovarian epithelium. The only study, to our knowledge, that has assessed ovarian cancer risk in a population with connective tissue disease was a relatively retrospective cohort study of patients with rheumatoid arthritis. Cibere et al. (141) examined the observed versus expected rates of numerous cancers among a cohort of 862 Canadian patients with rheumatoid arthritis followed for a mean of 17.4 years. Only five patients developed ovarian cancer, for a standardized incidence ratio of 0.89, which was not statistically significant. Although the number of observed cases was somewhat lower than expected, the number of cases was far too limited for clear interpretation. Larger studies would be of great interest.

Experimentally induced inflammation of the epithelial ovarian surface should be studied to see whether such manipulation will result in epithelial inclusion cysts. Furthermore, demonstration of markers of mutagenicity within inclusion cysts should be sought to suggest movement along a pathway toward ovarian cancer. For example, known markers of mutagenesis, such as mutations in tumor suppressor genes, if they are more common in inflammation-induced inclusion cysts, would provide evidence supporting the role of inflammation in ovarian cancer pathogenesis. Animal experiments could also examine whether suppression of ovarian epithelial inflammation with anti-inflammatory medications would reduce the number of inclusion cysts and the rate of cancer-associated mutations. Antioxidants may also lower ovarian cancer risk, and evaluation of such an effect in both animals and humans would be helpful in testing the inflammation hypothesis.

Susceptibility to the effects of ovarian epithelial inflammation may be modulated by DNA excision and repair potential; i.e., individuals with more precise or active DNA repair capabilities may be relatively spared from the effects of local inflammation. The prevalence of such DNA polymorphisms within women with ovarian cancer and control subjects could be tested. All of these are testable hypotheses that could help in our understanding of the biologic mechanisms underlying ovarian cancer.

It is likely that hypotheses regarding ovulation, gonadotropins, and inflammation are not mutually exclusive but are instead interactive. The occurrence of inflammation during ovu-

lation has been discussed. Steroid hormones may also mediate inflammation. Estrogens, according to the gonadotropin hypothesis, elevate ovarian cancer risk and they may also stimulate the immune response (142). In particular, estrogens have been demonstrated *in vitro* to stimulate B-cell response and decrease suppressor T-cell reactivity, resulting in elevations in antibodies and autoantibodies. Moreover, oral contraceptives elevate the concentrations of local immunoglobulin G and immunoglobulin A in the female genital tract (143). Elevated LH may also enhance oxidative stress. The principal bioassay for LH is the ovarian ascorbic acid depletion assay. Ascorbic acid is an antioxidant, and it is possible that LH depletes ascorbic acid by generating the production of free radicals (144). This observation—that a gonadotropin and estrogen may stimulate inflammation and oxidation—provides a link between steroid hormone excess and the physiologic events involved in inflammation. Thus, it is not necessary to argue as to whether the data fit one hypothesis better than another, but rather it is necessary to develop a more comprehensive model of pathogenesis that may incorporate a role for steroid hormones, ovulation, and inflammation in ovarian cancer. Such a model would account for epidemiologic data suggesting associations between reproductive factors and ovarian cancer and also between PID, endometriosis, talc and asbestos exposure, tubal ligation, and hysterectomy and ovarian cancer.

SUMMARY

Neither incessant ovulation nor gonadotropin stimulation of ovarian estrogen provides a completely satisfactory explanation for the genesis of ovarian cancer. We have reviewed the data suggesting that an additional mechanism that may underlie ovarian cancer is inflammation, with concomitant rapid DNA turnover and defective repair, oxidative stress, and elevation of bioactive substances. Incessant ovulation, a process that has been linked to ovarian cancer risk, is associated with inflammation at the level of both the epithelium and the follicle. Other factors that cause local pelvic inflammation may also increase risk. Finally, tubal ligation and hysterectomy, which diminish the potential that ovarian epithelium will be exposed to initiators of inflammation, reduce risk. Further observational and experimental data will be needed to confirm the hypothesis that inflammation is a central biologic process in ovarian cancer risk.

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NOTE

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Exhibit 107

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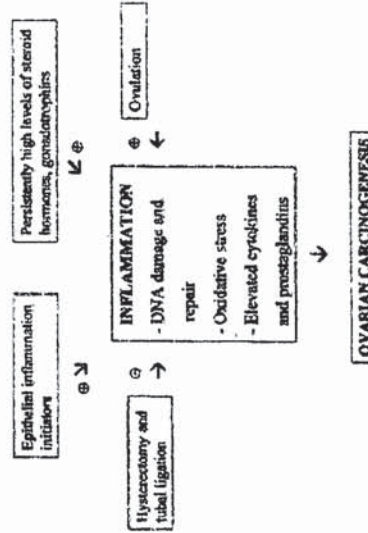
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DATE: September 30, 2004	FROM: Richard J. Zazenski Director Product Safety
TO: Bill Ashton	PHONE: 303-643-0404
	E-MAIL: rzazenski@luxemac.com
	FAX: 303-643-0446
cc:	Number of Pages: 13 Pages (including Cover Sheet)

Bill - I came across this paper this morning published in the April, 2004 journal "Human Reproduction", an official journal of the European Society for Human Reproduction and Embryology. It offers some compelling evidence in support of the "migration" hypothesis. Combine this "evidence" with the theory that talc deposition on the ovarian epithelium initiates epithelium inflammation -- which leads to epithelium carcinogenesis -- and you have a potential formula for NTP classifying talc as a causative agent in ovarian cancer.

Please note that the tables and figures cited in the paper are "pasted" after the References at the end of the paper.



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No. 3879 P. 2

Retrograde migration of glove powder in the human female genital tract

A.C.F. Sjösten¹, H. Ellis² and G.A.B. Edelman¹

¹Karolinska Institutet, Department of Obstetrics & Gynaecology at Stockholm Söder Hospital, S-118 83 Stockholm, Sweden and ²Department of Anatomy, Guy's, King's and St Thomas' School of Biomedical Sciences, London Bridge, London SE1 9RT, UK. To whom correspondence should be addressed. e-mail: anette.sjosten@karolinska.se

Abstract

BACKGROUND: This study in humans was undertaken to evaluate earlier results from animal research showing a retrograde migration of glove powder from the vagina into the intra-abdominal cavity. **METHODS:** One study group was gynaecologically examined with powdered gloves the day before an abdominal hysterectomy and another group 4 days pre-operatively. There were two control groups similarly examined with powder-free gloves. Cell smears were taken from the peritoneal fluid and during the operation further smears were taken from the Fallopian tubes, uterine cavity and cervical canal. **RESULTS:** Statistically significant differences were found for large starch particles at all locations between the study and control groups examined 1 day pre-operatively. Considering small starch particles, there were significant differences in cervix ($P < 0.001$), uterus ($P < 0.01$) and the Fallopian tubes ($P < 0.01$). The combined results also show significant differences between both large and small starch particles in cervix, uterus and the Fallopian tubes. There were also differences between the study and control groups examined 4 days pre-operatively, but these were not statistically significant except for small and large starch particles in uterus ($P < 0.01$, $P < 0.05$) and cervix ($P < 0.05$, $P < 0.05$). **CONCLUSIONS:** This study has pointed out a retrograde migration of starch also in humans after a gynaecological examination with powdered gloves. Consequently, powder or any other potentially harmful substance that can migrate from the vagina should be avoided.

Key words: female/gloves/retrograde migration/starch particles/vaginal examination

Introduction

Earlier case reports suggest that intra-abdominal granulomas or adhesions due to starch particles were caused by starch powder used on gloves during vaginal examination. An initial indication of retrograde flow through the Fallopian tubes was the finding of intraperitoneal starch granulomas (Paine and Smith, 1957*). Later the first case of starch peritonitis in a patient without previous surgery was reported (Saxen *et al.*, 1963*). A recent investigation detected talcum particles on the ovaries in women who had used perineal talc applications (Heller *et al.*, 1996*). In contrast, tubal ligation prevents the

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access of mediators that reach the peritoneal cavity through the Fallopian tubes (Ylikorkala, 2001*).

Powder-free gloves have been available for 20 years, but starch-powdered gloves are still available and in use (Sjösten *et al.*, 1999*).

It is well documented that starch-powdered gloves are not appropriate for abdominal surgery (Ellis, 1990*; Holmdahl *et al.*, 1994*), and intraperitoneally, starch particles can initiate inflammatory reaction and the formation of adhesions (Edelsiam *et al.*, 1992*; diZerega, 1994*), although the mechanism by which starch increases the propensity of tissues to form adhesions is not known. Reduced peritoneal fibrinolysis and activation of leukocytes by particulate starch granules have been suggested as possible mechanisms. Activated leukocytes, particularly macrophages, produce supernormal amounts of oxygen-free radicals, prostaglandin E₂, thromboxane B₂, and various cytokines (Osman and Jensen, 1993*). Starch particles also increase the eicosanoid production which may contribute to the inflammatory or immune reactions and development of adhesions (Chagini and Rong, 1999*). If already injured mesothelial surface of the peritoneum is exposed to starch, more dense adhesions are created compared to the effect of peritoneal trauma or starch separately. Application of glove powder on minimally or severely traumatized peritoneum facilitates tumour cell adhesion and growth alone (van den Tol *et al.*, 2001*). Histological re-evaluation after tubal reconstructive surgery due to peritubal or peri-ovarian adhesions has shown residual starch from powdered gloves (Yaffe *et al.*, 1980*).

A causal connection has been shown between operative tissue damage, intra-abdominal ischaemia, infections, reactions to foreign materials such as sutures, particles of gauze, glove dusting powder and post-operative adhesions (Mylämiemi, 1967*; Holmdahl *et al.*, 1996*). One of the proven causes of post-operative intestinal adhesions is microscopic foreign bodies which are present in up to 93% of adhesions (Duron *et al.*, 1997*). After open abdominal or pelvic surgery, a third of the patients are readmitted at least twice during the subsequent 10 years for a disorder directly or possibly related to adhesions (Ellis *et al.*, 1999*).

Our previous investigation in a rabbit model indicated a retrograde migration of glove powder from the vagina into the intra-abdominal cavity (Edelsiam *et al.*, 1997*). The amount that reaches the peritoneum is sufficient to significantly increase formation of post-operative adhesions after a standardized trauma (Sjösten *et al.*, 2000*).

Therefore, this subsequent study in humans was done to investigate whether starch particles from powdered gloves also in humans might gain access to the abdominal cavity through the vagina after a gynaecological examination with powdered gloves.

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A considerable number of gynecologists wears starch-powdered gloves (Sjösten *et al.*, 1999*), despite evidence of starch-induced complications. The starch particles can migrate not only from the vagina into the cervical canal and the uterine cavity but also through the Fallopian tubes into the peritoneal fluid. Women exposed to intra-abdominal surgical trauma 1-4 days after a gynecological examination with powdered gloves may be at increased risk of intra-abdominal adhesions. But even without a surgical procedure there is a risk of intra-abdominal or peri-tubal adhesions due to the examination with powdered gloves (Osser *et al.*, 1989*). Ongoing subclinical PID can cause infective tissue damage. An extensive study by Myllärmi (1967*) showed that talc, starch powder and lint in the abdominal cavity tended to accumulate in the traumatized areas of the peritoneum so that the foreign material contaminating the peritoneal tissues could act together with other traumatizing conditions, possibly preventing the resorption of fibrinous adhesions. This corresponds to our previous finding in the rabbit model that starch particles deposited in the vagina can migrate in a retrograde direction from the vagina into the abdominal cavity and, combined with an intra-abdominal trauma, generate dense adhesions (Sjösten *et al.*, 2000*). Since there are indications towards retrograde migration of powder, it must not be used regardless of cyclic variations or sexual activity.

In conclusion, our results show that starch particles can migrate from the vagina into the cervical canal, the uterine cavity and through the Fallopian tubes up to 4 days after a gynecological examination with powdered gloves. Glove powder contributes to adverse intra-abdominal reactions, which include adhesion formation and adhesion-related complications such as chronic pelvic pain and bowel obstruction. Tubal and pelvic adhesions are a major cause of female infertility. Since evidence suggests that a retrograde migration could be a general mechanism, our recommendation is that we should be critical of harmful substances, e.g. glove powder, that could migrate from the vagina to abdominal cavity.

► Acknowledgements

We thank Associate Professor Mr Göran Grauth for statistical analyses. This study was supported by Karolinska Institutet, Stockholm, Sweden.

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Statistics

Non-parametric Mann-Whitney U-tests and Fisher's exact test were used and values are given as SEM for the group. Differences were considered significant at the $P < 0.001$, $P < 0.01$ and $P < 0.05$ levels. All statistical tests were computerized and carried out with statistics programs (Statistica[®], Statsoft, USA).

Results

Group I: examined 1 day pre-operatively with (i) powdered gloves (n = 17) and (ii) powder-free gloves (n = 15)

Starch particles were found in the cell smears with more particles found on the slides from the patients examined with powdered gloves. The differences were significant at all locations in the genital tract for small particles (cervix $P < 0.001$, uterus and Fallopian tubes $P < 0.01$) and large particles (cervix and uterus $P < 0.01$ and Fallopian tubes $P < 0.05$) but only for large particles in the peritoneal fluid ($P < 0.05$). However, in two patients examined with powdered gloves, no particles were found. On the contrary, in three patients examined with powder-free gloves, a few particles were found (Table I and Figure 1).



Figure 1. Median and range value for the retrograde transperitoneal of small and large starch particles respectively, in different locations 1 day after a gynaecological examination with or without powdered gloves. The negative range value in the starch group for cervix, uterus and peritoneal fluid are due to contamination with airborne starch particles.

Group II: examined 4 days pre-operatively with (i) powdered gloves (n = 12) and (ii) powder-free gloves (n = 14)

There were significantly more small starch particles as well as large particles (cervix and uterus $P < 0.05$) after examination with powdered gloves. The differences were the same for small particles but less significant for large particles (uterus $P < 0.05$). The differences were non-significant in the Fallopian tubes and the peritoneal fluid (Table II and Figure 2).

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► Materials and methods

Patients

The participants in the study were divided into four different groups. Informed consent was obtained from all participants. All had a routine gynaecological examination before an elective laparotomy for total or subtotal hysterectomy due to fibroids or menometrorrhagia. Group I: examined 1 day pre-operatively with (i) powdered gloves (Gammex® Ausell GmbH, Germany; $n = 17$, mean age 51 years) or (ii) powder-free gloves (Biogel® Regent Medical, SLL) ($n = 15$, mean age 51 years). Group II: examined 4 days pre-operatively with (i) powdered gloves ($n = 12$, mean age 53 years) or (ii) powder-free gloves ($n = 14$, mean age 52 years). Patients with cancer of the uterus were excluded as well as women with ongoing menstrual bleeding. The pre-menopausal women were examined regardless of the follicular or luteal phase of the menstrual cycle. A third of all women in the study were post-menopausal. Any medication that might have influenced the tubal patency had not been taken except in the case of three patients who had an asthmatic disease and needed to take terbutaline occasionally. The medication was not taken during the investigations. There were no other significant differences for patient characteristics. Sexual activity, cyclic changes or hormonal effect were not considered in this study.

Surgical procedure

An abdominal subtotal or total hysterectomy was undertaken with the operating team and the nurse who set up the instrument tray wearing powder-free gloves. Immediately the abdominal cavity was opened, peritoneal fluid was collected and cell smears were then taken from the peritoneal fluid. From the fimbriae of the Fallopian tubes, additional cell smears were taken pre-operatively and when the uterus had been removed, i.e. post-operatively from the uterine cavity and the cervical canal. For making the smears sterile, forceps or pens were used. Smears from the fimbriae of the Fallopian tubes were omitted if they were not removed during the hysterectomy.

Cell smears

The cell smears were quantitatively standardized on $\sim 1 \text{ cm}^2$ of one-half of a glass slide with the other blank side serving as control for contamination with air-borne starch particles. All the slides were stained with May-Grünwald Giemsa by a biochemical assistant wearing powder-free gloves in a laboratory where only powder-free gloves were used. The slides were coded and analysed by two independent investigators with a Zeiss 476 microscope using polarized light at magnification $\times 250$. The starch particles were counted in a standardized procedure for all slides. The numbers on the blank side (i.e. contamination) were subtracted from that in the smears so that the number of starch particles on each slide represent the net number without contaminating particles. Since there are differences in the size of starch particles they were divided into two sizes: (i) smaller than a leukocyte and (ii) larger than a leukocyte. Leukocytes for comparison in size were always present in the smears. The study was approved by the local ethics committee.

Exhibit 108

Review

Open Access

Peritoneal inflammation – A microenvironment for Epithelial Ovarian Cancer (EOC)

Ralph S Freedman*¹, Michael Deavers², Jinsong Liu² and Ena Wang³

Address: ¹Department of Gynecologic Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA, ²Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA and ³Department of Transfusion Medicine, NIH, Bethesda, MD, USA

Email: Ralph S Freedman* - rfreedma@mdanderson.org; Michael Deavers - mdeavers@mdanderson.org; Jinsong Liu - jliu@mdanderson.org; Ena Wang - EWang@cc.nih.gov

* Corresponding author

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Abstract

Epithelial ovarian cancer (EOC) is a significant cause of cancer related morbidity and mortality in women. Preferential involvement of peritoneal structures contributes to the overall poor outcome in EOC patients. Advances in biotechnology, such as cDNA microarray, are a product of the Human Genome Project and are beginning to provide fresh opportunities to understand the biology of EOC. In particular, it is now possible to examine in depth, at the molecular level, the complex relationship between the tumor itself and its surrounding microenvironment.

This review focuses on the anatomy, physiology, and current immunobiologic research of peritoneal structures, and addresses certain potentially useful animal models. Changes in both the inflammatory and non-inflammatory cell compartments, as well as alterations to the extracellular matrix, appear to be signal events that contribute to the remodeling effects of the peritoneal stroma and surface epithelial cells on tumor growth and spread. These alterations may involve a number of proteins, including cytokines, chemokines, growth factors, either membrane or non-membrane bound, and integrins. Interactions between these molecules and molecular structures within the extracellular matrix, such as collagens and the proteoglycans, may contribute to a peritoneal mesothelial surface and stromal environment that is conducive to tumor cell proliferation and invasion. These alterations need to be examined and defined as possible prognosticators and as therapeutic or diagnostic targets.

The peritoneum and its structures are integral to the microenvironment of epithelial ovarian cancer (EOC). The peritoneum comprises a single layer of mesothelial cells at the surface, covering abdominal organs (visceral or serosal layer) and the abdominal and pelvic wall (parietal layer or peritoneum).

About 80% of the more common epithelial ovarian cancers (EOC) involve the peritoneum or serosal surfaces as

microscopic foci and visible lesions. The metastases may be exophytic with direct exposure to the peritoneal cavity and its contents or subperitoneal foci coalescing over time to form variably sized plaque-like deposits (Figure 1). Involvement of the peritoneum predicates an adverse situation for the patient that impacts significantly on prognosis as evidenced by the fact that Stage I patients have a 5 and 10 year survival of 90% [1], whereas patients with Stages III and IV disease have a 5 year survival of about

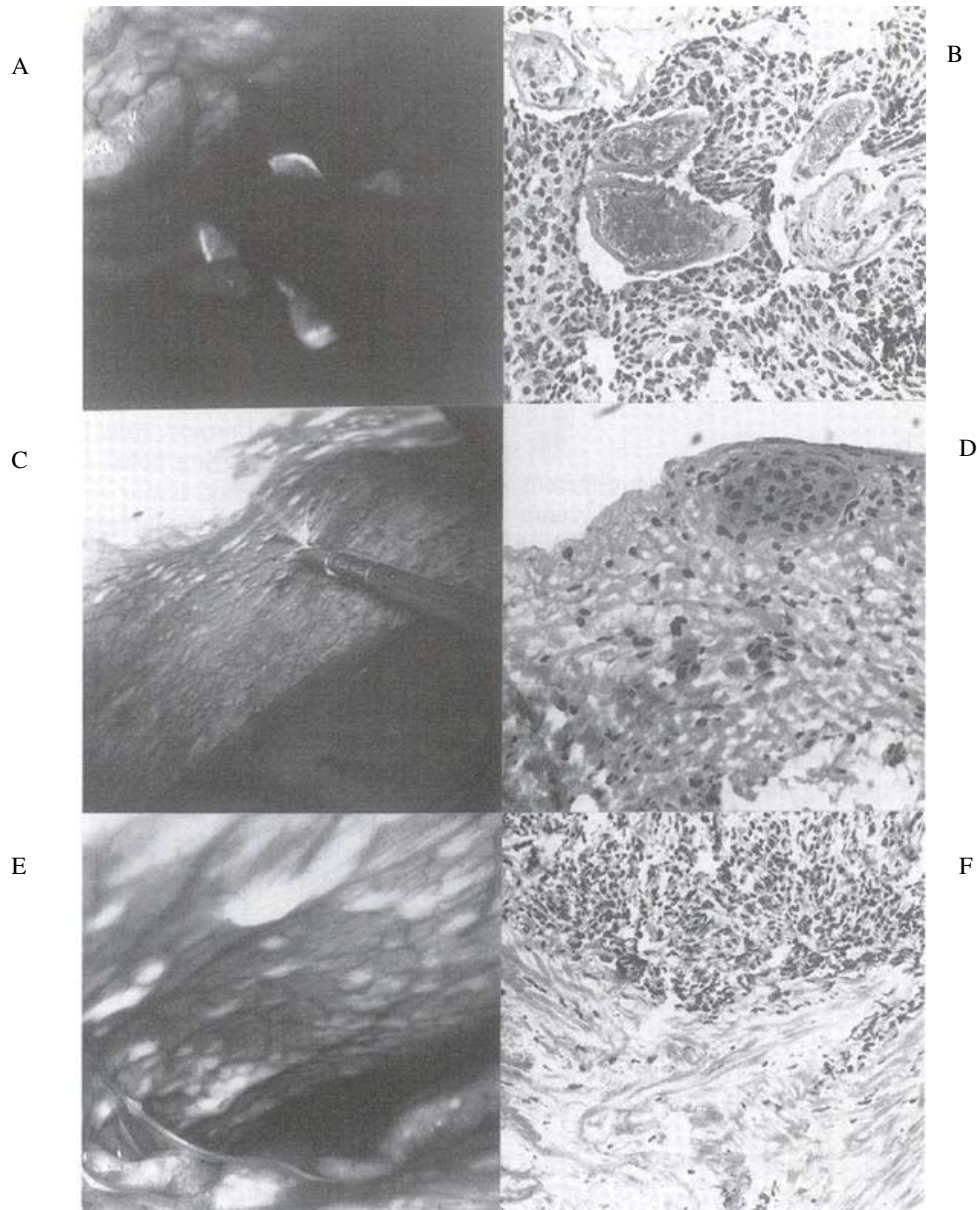


Figure 1

Surgical restaging by laparoscopy (peritonoscopy) and histopathologic findings showing different patterns of peritoneal involvement following prior systemic chemotherapy. (A,B) Exophytic peritoneal metastases approximately 1 cm in diameter showing multiple capillary loops. Histologic evaluation demonstrates numerous blood vessels surrounded by tumor cells. (C,D) Small 1 mm sized peritoneal metastases that are subperitoneal on histologic evaluation. (E,F) Multiple metastases about 1 cm in diameter that are growing deep to the peritoneal surface and coalescing to form plaques. Histologic evaluation demonstrates that these lesions are relatively avascular and contain significant amounts of peritumoral fibrosis. (Re-printed with permission from *Cytokines, Cellular & Molecular Therapy*).

20%. Though most patients presenting with advanced disease show an initial response to chemotherapy, their fates are ultimately dependent upon sensitivity or resistance to chemotherapy agents or other factors. The important contributions of the tumor microenvironment to the malignant phenotype has been demonstrated in recent preclinical tumor models [2-4]. Findings from a recent study of human EOC tumors also suggest the possibility of genomic instability in nontumor tissues adjacent to growing tumor foci in EOC patients [5]. The current review summarizes the structural and functional components of the peritoneum, which could facilitate tumor progression and metastasis.

Anatomy and physiology of the peritoneum

The peritoneum has the structure and functions of an organ that is organized for the protection of the integrity of other abdominal organs and viscera. The surface epithelium of the serous membrane of the peritoneum and serosa, is attached to a basement membrane lying on a stroma of variable thickness, and is comprised of collagen-based matrix, blood vessels, lymphatics, nerve fibers, and, in the normal state, rare hematogenous cells. A detailed description of the micro and ultra structural anatomy is described elsewhere [6,7]. The structural and functional configuration of the peritoneum allows for an important homeostatic role through rapid mobilization of inflammatory mechanisms that can efficiently localize an injury or infection. The peritoneal surface layer has spaces or stomata between the surface mesothelial cells that could readily allow transfer of molecules or possibly cells between the stroma and the peritoneal cavity, or vice versa.

Immunohistochemically documented structures in the submesothelial layer include Type I and III collagen, fibronectin, elastin, and laminin at the basement membrane stromal interface and glycosaminoglycans [6]. Epithelial inclusions, referred to as endosalpingosis, can occur, though its cause is unknown. Ultrastructurally, both tight junctions and intercellular spaces are present. The presence of these junctions can affect the transfer of particles or cells. Molecules may transit either across or between the cells to the stromal compartment and vice versa.

Pathology and altered function of peritoneum in EOC

Peritoneal and serosal seeding is a frequent occurrence in EOC, but there is little known about the role of the multi-structured peritoneum in contributing to invasion, metastasis, and tumor proliferation. It is possible, if not probable, that critical alterations in the peritoneum surface and stroma precede either lymphatic or hematogenous spread to distant sites. In EOC patients, there may be substantial alterations to the peritoneum both at the macroscopic and

submacroscopic levels. Such alterations may include thickening of the surface membrane with or without malignant ascites formation and overtly enhanced vascularity. In certain patients, the peritoneum may have a florid appearance of peritonitis with edema, enhanced vascularity, and soft adhesions. At the microscopic level, there may be multilayering of the surface epithelium (hyperplasia) and an inflammatory infiltrate comprised of different leukocyte populations. Retroperitoneal fibrosis can be extensive and can interfere mechanically with anatomic structures in extraperitoneal locations, including ureters, lymphatics, and the bowel in different locations.

It would appear that a reorganization of the collagen-based matrix associated with the malignant process in EOC patients might accompany an inflammatory cell reaction. This could be similar to the situation in the peritoneum of renal fibrosis, which results in hyperplasia of the surface layer and extensive macrophage infiltrates into the stroma [8,9]. Since the peritoneal and serosal membranes lie in proximity to the primary tumor or its metastases, the question could be asked whether soluble products of tumor masses and nodules might transfer to the normal surface mesothelial cells and penetrate the subjacent stromal tissues. Molecules such as cytokines or chemokines released from the tumor into the peritoneal cavity could possibly prime these tissues for tumor spread, proliferation, and metastasis. The peritoneum can easily permit transperitoneal passage of molecules, even up to the size of albumin, and, depending on their adherence and reactivity with stromal compartment structures, might either transfer to the capillary bed or accumulate in the subperitoneal compartment, with later entry to the lymphatics. The dynamics of molecule transfer across the peritoneum as it applies to intraperitoneal therapy pharmacology and pharmacodynamics are reviewed elsewhere [10]. Peritoneal membrane and stromal structures exhibiting adherence properties for such molecules or cells, however, might retard their removal from this site, contributing to a sensitizing effect on the peritoneum.

Dual role of the inflammatory reactions in EOC

There is substantial data to support the presence of immune cell infiltration in EOC and its microenvironment. In earlier studies, we had shown that T cells comprised about 70% of mononuclear leukocytes in solid EOC tumors [11], and results from a number of experiments by us and by others suggested that the presence of these T cells could be associated with an antigen-driven immune response [12-17]. This effect is supported by the presence of clonally expanded T cell transcripts in ovarian TIL [18]. Notwithstanding these findings, we [19] and others [20] have found little evidence for the presence of an active ongoing adaptive immunity *in vivo*. This is

supported by the absence of IFN γ transcripts in solid tumors and their infrequent detection in ascitic T cells [19]. Others have also reported absent CD3 TCR ζ on TIL [20], and absent or low levels of IFN γ protein detected in ascites of EOC [21]. It is possible that cloned T cells in the tumor environment could represent tolerized cells, though antitumor activity can be generated *ex vivo* when these T cells are exposed to appropriate activation stimuli [13,14]. The presence of regulatory T cells [22], and certain macrophages [23], which are producers of IL6, IL10, and TGF β could favor an immunosuppressive environment and may contribute to tumor progression and metastases [24]. The role of IL10 remains to be elucidated as, depending on the status of the tumor, this cytokine can either enhance or suppress immune responses [25].

Large numbers of monocyte/macrophages (MOMA) are also present in ascitic fluid where they may comprise 50% or more of the mononuclear leukocyte population, whereas the proportion of T-lymphocytes is usually below 40% [11]. In recent preliminary studies, we have found that pelvic peritoneal biopsies from advanced stage EOC patients, even in the absence of tumor involvement of the specimens, also has a high proportion of MO/MA. The MO/MA in EOC comprise several subsets with the notable presence of CD14+DR- and CD14+DR+ CD16+ cells [26]. The differentiation potential and functional capacity of these MO/MA in cancer patients is largely undetermined but clearly there are differences in the phenotypic characteristics between normal and EOC patients [26]. Inflammatory infiltrates have long been observed in human cancer tissues, but their significance in the non-lymphomatous solid tumors has largely been ignored by pathologists and clinicians. There is increasing recognition that infiltrating immune cells may contribute to either enhancement of immunity or tumor growth and progression [24]. Both MO/MA and T cells may have this dual role, and it remains a challenge to steer the activity of these populations toward an effective antitumor response *in vivo*.

We have recently employed a custom-made standardized cDNA microarray that utilizes probes for 16,500 genes to conduct a pilot study on the peritoneum of patients with EOC [27]. Biopsies were obtained at the time of peritoneal entry on patients undergoing exploratory surgery for suspected EOC. For controls, we utilized specimens of parietal peritoneum obtained at the same sites from patients undergoing surgery for suspected benign disease. Results from this study showed that the superficial layer, including the surface peritoneum and subjacent stroma specimens from the malignant group, revealed unique features at the transcript level compared to the benign group. These features are characterized by a dynamic process including cell attachments, signaling, growth stimula-

tion, and, most importantly, a proinflammatory, pro-angiogenic, and extracellular matrix (ECM) remodeling effects. The peritoneum and subperitoneal stroma from the benign cases showed homogeneity in their transcript expression without the proinflammatory signature contrasting with some heterogeneity from patients with EOC, but an emphasis on inflammatory network responses and cell infiltrates.

Cytokines and chemokines as facilitators of a protumor microenvironment

With increased knowledge in endothelial attachment and transcapillary migration, there is now a focus on inflammatory as well as non-inflammatory cell infiltrates and their contribution to cancer cell spread. Chemokines and certain of the larger cytokines may contribute to the migration of leukocytic and other cells into a tumor environment among their other properties. The chemokines now have a new nomenclature based on their chemical structure [28], and extensive reviews have been published [29]. In EOC, particularly in studies on ascites, substantial amounts of certain CC and CXC chemokines have been demonstrated, including CCL18 (PARC), CXCL8 (IL8), CCL2 (MCP1), and CCL3 (MIP1 α) [30] (Table 1). Transcripts for CCL4 (MIP1 β), CCL5 (RANTES), CCL7 (MCP3) have been demonstrated in EOC cells [31] CCL13, however, is produced by ascitic macrophages and cannot be induced in EOC cells [30]. Chemokines and cytokines may have in common potent functional properties, such as chemotaxis and proangiogenesis, and typically have effects in proximity to cells producing them. Larger cytokine molecules, such as TGF β , may also have chemotactic and proangiogenic effects. In advanced disease, tumor cells and other cells of nontumor origin, can contribute to chemokine production. CXCL8 (IL8) is very pleiotropic and is constitutively produced or induced by both hematogenous and non-hematogenous cells and by hypoxia. We found that CXCL8 was overexpressed on the peritoneal stroma along with other network genes and appears to be a pivotal chemokine with substantial interactions at the transcript level with genes that are involved in inflammation, angiogenesis, and chemotaxis [27,32]. Receptors for the chemokines are expressed on a variety of hematogenous cells, including T cells and macrophages [33]. Of interest, CXCR4, the receptor for CXCL12 (SDF1), appears to be selectively expressed on EOC cells [34] and may contribute to tumor cell migration. There is a lack of detectable change in expression of other chemokine receptors in response to cytokines, except for CCR2, the receptor for CCL2 and certain other CC chemokines, which appears to be downregulated on EOC ascitic macrophages [35]. This effect may interfere with migration of macrophages away from the tumor site while contributing to a tumor-promoting environment [35]. Unlike cytokines, many chemokines may exhibit more

Table 1: Chemokines/Receptors in EOC

Ligand (Alt. Name)	Receptors	Cells Targeted	Correlates in EOC
CCL2 (MCP1) *+	CCR2	Activated T, Monocytes, DC, Basophils	CD8 ⁺ T cells, CD68 ⁺ MA ↓ on ascitic MA
CCL3 (MIP1 α)*+	CCR2	Activated T, NK, MO, Eosinophils	
CCL4 (MIP1 β)*+	Unknown		
CCL5 (RANTES) *	CCR2	Activated T, NK, MO, Eosinophils	
CCL7 (MCP3) +	CCR2		
CCL18 (PARC) +	Unknown		MA produced but not induced in EOC cells
CXCL8 (IL8) *+	CXCR1, CXCR2	Neutrophils, Resting T	
CXCL12 (SDF1) *+	CXCR4	Neutrophils, Resting T, Activated T, B, MO	CXCR4 preferentially expressed on EOC cells

* In RNA detected on EOC cell lines + Proteins detected in ascites

promiscuous binding to receptors. This may insure a regional effect through their redundancy.

Several cytokines have been detected in serum and ascites of EOC patients, including TGF β isotypes, IL10, IL6, TNF α , CSF1 and IL1 [19,36,37,12,38]. TGF β isotypes are produced by EOC cells [39] on mononuclear leukocytes, including CD14⁺DR⁻ [23] and T regulatory cells [22]. TGF β , in its activated form, was previously considered a tumor-inhibitory cytokine but its tumor-reactive properties appear to be more complex (Table 2). TGF β also can have a tumor promoting effect in advanced cancer possibly through activation of cdk inhibitors that block the unbinding of the pRb/E2F transcripts [40], and interference with TGF β receptor binding mediated by H-Ras, as well as consequent to c-myc, its reaction with the E2F transcription factor complex [40]. The signaling pathway of TGF β within tumor cells may also be subverted due to mutations, or interactions with other cytokines. A TGF β activation response might, however, prevail in the micro-environment where it may contribute to myofibroblast and endothelial cell chemotaxis, tumor adhesion, and suppression of adaptive and innate immunity [41]. IL10 is also produced in association with EOC with a large contribution by CD14⁺DR⁻ MO/MA, and these cells may function as immune regulatory cells. IL6 is expressed by EOC tumor cells as well as mesothelial cells and has been detected in the serum and ascites of EOC cells [42-45]. A recent study has shown that IL6 and MCP production by submesothelial cells can be enhanced during abdominal surgery [46]. IL6 also enhances tumor attachment and proliferation of tumor cells, most likely through the PI 3-K activation mechanisms, and can interfere with the maturation of MO/MA to DC [44,45]. This finding might contribute to the large number of functionally immature DC in the ascitic fluid and absent levels of IL12, a product of DC maturation [47,48] (and C Butts' unpublished observations).

Factors associated with composition and decomposition of the extracellular matrix (ECM)

Phenotypic and functional characterization of stromal inflammatory and non-inflammatory cell infiltrates will be useful for understanding the biology of metastasis. These infiltrates probably occur following transcapillary migration. In this respect, the chemical composition and dynamics of the extracellular matrix (ECM) are also likely to be important. Thus, chemokines may "stick" to other proteins in the stromal microenvironment, enhancing their chemoattraction and other properties by accumulating at these sites. Proteoglycans, which comprise a protein core, and sulphated or non-sulphated aminoglycan side chains could facilitate this. The proteoglycans include a variety of molecules, such as versican, decorin, hyaluran, and heparan with different side chains. The side chain of decorin can be of the dermatin type or chondroitin SO₄ type, each having non-overlapping different functions. We have previously shown that decorin chondroitin SO₄ is expressed with myofibroblasts in the adjacent stroma of EOC tissues [49] (Figure 2). A recent paper has shown that endothelial cells stimulated in culture on a collagen type I matrix in the presence of IL6 and IL10 synthesized decorin [50]. This is of particular interest since both IL6 and IL10 are highly expressed in EOC. Chemokines may attach covalently to proteoglycans that express GAG sequences, while retaining their effects on tumor microenvironment cells. This may facilitate their effects locally. In contrast, proteoglycans might also interfere with the binding of activated TGF β to its receptors.

A large family of receptors called integrins can regulate many functions at both the cellular and ECM levels. The integrins are important for the spread and proliferation of cancer cells [2]. Their functions, however, are complex since integrins can associate with other integrins or growth factor receptors or adaptive proteins producing bi-directional effects to and from the cell membrane surface. Integrin mediated effects include cytoskeletal changes through complexing with α actinin and other proteins with downstream effects on actin. These changes may

Table 2: Dual Effects of Cytokines on Tumor/Tumor Microenvironment

TGFβ	IL6
<ul style="list-style-type: none"> • TGFβ + TGFβ RIII → TGFβ - RII + RI heterodimer → TGFβ RI - P + SMADs → SMAD - P → nucleus → initiates transcription • TGFβ → in repression cell cycle genes or activation • Repression involves activation of cycle dependent kinase inhibitors, blocks unbinding of pRb/E2F transcripts • Other interactions include: H-Ras (↓ RI & ↑ RII); C-Myc (stimulates proliferation by repressing cdk inhibitors) associates w/E2F transcript factor complex • TGFβ effect negated by disruption of signal pathway • Alterations to the microenviroment Tumor adhesion Endothelial chemotaxis (proangiogenic) Myofibroblast chemotaxis • Immunosuppressive Effects Adaptive ↓ MHC expression (targeting) ↓ Costimulatory Ag expression by DC Blocks pre CTL → CTL Suppresses TH1 cells - Shift to TH2 Induces apoptosis Suppresses proliferative response to APCs Innate Cells Inhibits NK & MA activation 	<ul style="list-style-type: none"> • ↑ TuC attachment migration • Immune modulation (T-cell ↑) • Interferes w/MA maturation to DC • Proliferation thru PI3-K activation <p>IL10</p> <ul style="list-style-type: none"> • ↓ MHC expression on TC • ↓ Costimulatory Ag expression • Suppress cytotoxic T-cell activation • Inhibits IFNγ production • Inhibits T-cell production

affect cell survival, proliferation, motility, and differentiation. Depending upon the particular signaling pathway dominance, the downstream effect may be either repression or activation of a particular function. Integrins may modulate or enhance the expression of other integrins and receptors, e.g. $\beta 1$ and $\beta 3$ integrins, which can influence apoptosis. Lack of detection of the cell-to-cell adhesion molecule E-cadherin on cuboidal cells of ovarian surface epithelium in contrast to its upregulated expression on metaplastic intra-ovarian cystic glands and early well-differentiated glandular carcinoma suggest that the latter cells may have been derived from the migrating surface ovarian cells [51]. E-cadherin also inhibits the anti-apoptotic PI3K signaling pathway and E-cadherin expression in advanced EOC metastatic nodules appears to be less prominent than other cadherin molecules [52]. In a 3-D model of breast cancer, chronic activation of the $\beta 1$ integrin has been shown to enhance the cancer phenotype in contrast to a different signaling effect from $\alpha 6/\beta 4$ integrin activation, leading to suppression of the cancer phenotype. In advanced EOC, the reduction or loss of E-cadherin expression is hypothesized to contribute to the spread and progression of the tumor [52]. E-cadherin in both breast and ovarian cancers is considered a late tumor suppressor molecule. The importance of $\beta 1$ signaling has been shown in experiments that demonstrate reversion of the malignant phenotype when $\beta 1$ integrin is blocked with anti $\beta 1$ mAbs [53]. These findings overall suggest that altered expression of adhesion molecules, such as cadherein, could serve different functions during the

pathogenesis of the EOC disease process. Antibody-mediated inhibition of integrin 1 has also been shown to interfere with production of decorin which is an important part of the ECM [50].

Animal models for EOC/peritoneal interactions

Representative animal models of EOC need to combine the oncogenic developmental pathways as well as contributions from the cellular and ECM environment of the epithelium and stroma. This relationship, however, is clearer in established virally induced tumors [4].

There has been some recent progress in the development of suitable mouse models for human ovarian cancer [54,55]. Because of marked heterogeneity of ovarian cancer both at histopathologic and clinical levels, the underlying mechanisms that produce the altered gene expression profile in the EOC is not clear. However, it is reasonable to assume that the altered gene expression profile is at least in part due to activation of oncogenic events that transform the ovarian surface epithelial cells. Toward this end, it has been shown that oncogenic HRAS^{V12} or KRAS^{V12} activates multiple proinflammatory cytokines and angiogenic factor cytokines during the malignant transformation of ovarian surface epithelial cells in a newly created genetically defined model for ovarian cancer. In this model, introduction of SV40 T/t antigen extended the life span of primary cultured ovarian surface epithelial cells for a few more passages; however, these T/t antigen-expressing cells are still mortal. Introduction of the cata-

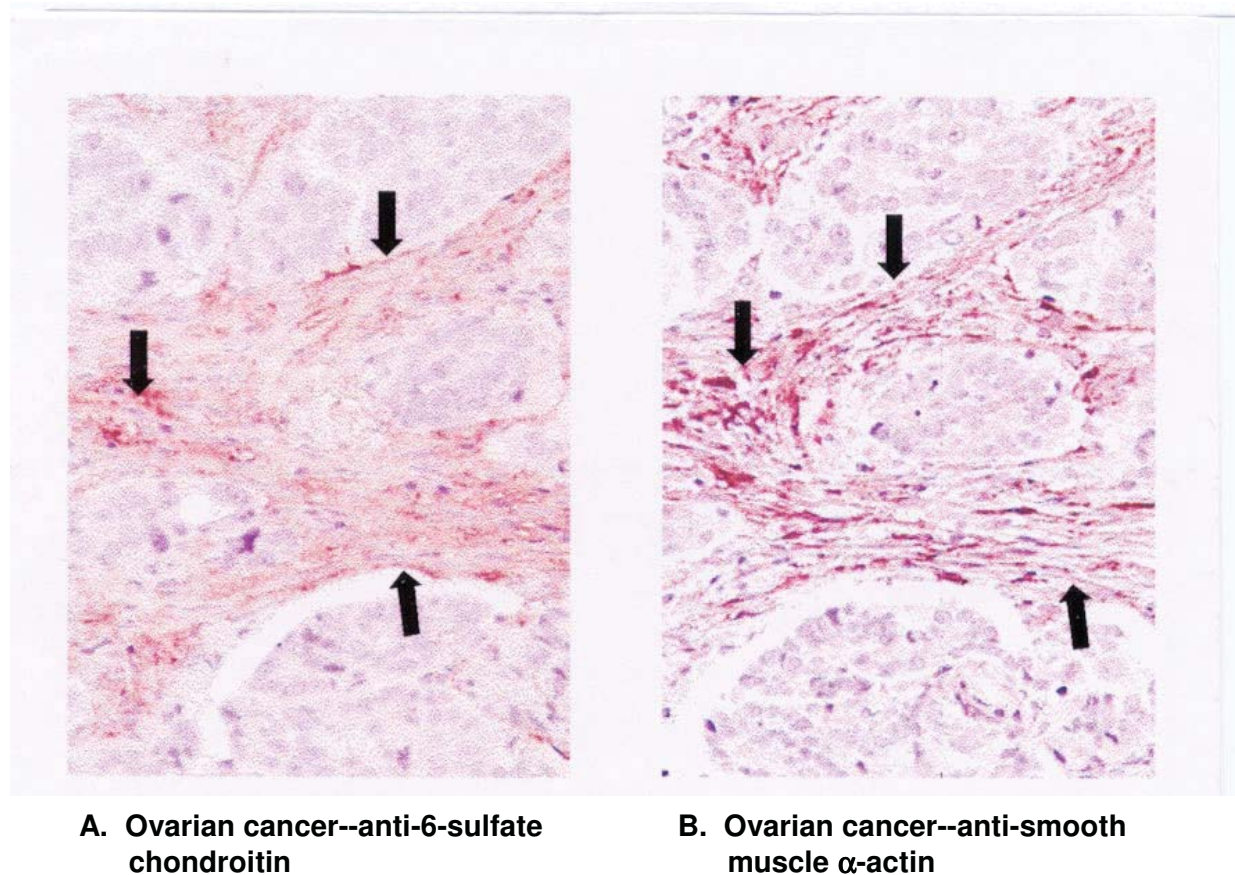


Figure 2

Histochemical staining of human ovarian tissue with anti-6-sulfate chondroitin or anti-smooth muscle α -actin antibody. Serial 5- μ m sections of paraffin-embedded tumors were stained with anti-6-sulfate chondroitin (A) or anti-smooth muscle α -actin (B). Arrows point to regions of staining overlap. (Re-printed with permission from *Clinical Cancer Research*).

lytic subunit of telomerase (hTERT) results in immortalization of these cells. Introduction of HRAS^{V12} or KRAS^{V12} results in transformation of these cells as reflected on the increased number of anchorage independent growth and tumor development after subcutaneous injection of these cells. Peritoneal injection of the transformed cells produced undifferentiated carcinoma or malignant mixed müllerian tumor and developed ascites, the tumor cells are focally positive for CA125 and mesothelin. Gene expression profile analysis of transformed cells revealed elevated expression of several cytokines including interleukin (IL)-1 β , IL6, and IL8, that are up-regulated by the NF- κ B pathway, which is known to contribute to naturally occurring human EOC. Incubation with antibodies to IL-1 β or IL8 led to apoptosis in the ras-transformed cells and

ovarian cancer cells but not in immortalized cells that had not been transformed. Thus, the transformed human ovarian surface epithelial cells recapitulated many features of natural ovarian cancer including a subtype of ovarian cancer histology, formation of ascites, CA125 expression, and NF- κ B-mediated cytokine activation. These cells provide a novel model system to study human ovarian cancer. Because of the remarkable similarity of gene expression between the RAS-transformed ovarian surface epithelial cells and peritoneum associated with ovarian cancer, these immortalized preneoplastic ovarian epithelial cell lines may provide a valuable experimental tool to examine the role of each of the cytokines in the peritoneum during ovarian cancer development [54].

Susceptibility of the stromal compartment of the peritoneum to proliferative signals has been well documented in animal models of the peritoneal fibrosing syndrome following exposure to chemical peritoneal dialysates [9,56]. In these models, alterations to the stromal environment occur in response to the dialysate resulting in infiltration of two main cell populations, fibroblasts secreting MCP-1, VEGF, and HSP47 and macrophages which can express TGF β , TNF α , IL1 and fibronectin. Macrophages in these models were shown to be recruited by several CC chemokines, MCP-1, RANTES, and MIP-1 and collagen-dependent endothelial cells. Moreover, removal of the fibroblast element in a knockout animal model abrogated the MA infiltration and the fibrotic process. A similar process has been described in renal fibrosis, which also involves mononuclear leukocytes and myofibroblasts. In EOC, it is possible that molecules derived from the primary or peritoneal surface metastases could be distributed throughout the peritoneal cavity. Even in the absence of ascites, the distribution of these molecules could possibly be facilitated by negative pressure in the peritoneal cavity and peristalsis of the intestines. The precise mechanisms underlying the formation of ascites is unknown. Ascites indicate a more advanced stage of the disease which could be a consequence of alterations in permeability of the peritoneum or extensive lymphatic obstruction. In subperitoneal metastatic growth, the ascites may be almost acellular whereas surface exophytic lesions may be accompanied by large numbers of free-floating tumor cells, mesothelial cells and leukocytes, and in some cases, the ascites has a hemorrhagic appearance.

In summary, we have shown that peritoneal structures of patients with EOC are different at the transcript level from those of patients with benign conditions [27]. The changes observed reflect alterations in cytoskeletal and signaling pathways that suggest regional activity from integrins, cytokines, hormone growth factors, and adaptive proteins. In addition, there appears to be intense chemokine activity, particularly of the CXC motif chemokines, suggesting a pattern of chemotactic influence on leukocytic as well as other cell types. Enhanced collagenase activity would contribute to remodeling of the stromal compartment and creation of a favorable environment for infiltration of leukocytes as well as other cells, such as myofibroblasts and endothelial cells. Gene profiling of the peritoneum may provide hints about early transition steps to cancer or provide insight into changes that may actually facilitate the spread of cancer to adjoining tissues. It is anticipated that future studies using high throughput technologies with a multidimensional approach will enhance understanding of these alterations and their biological significance. These efforts could help identify critical alterations in the environment surrounding the cancer and its metastases and might ultimately

lead to advances in diagnosis, prognosis, and novel approaches to therapeutic targeting in EOC.

The past few decades have seen considerable progress in chemotherapeutics of EOC that are contributing to an overall reduction in mortality [57]. However, EOC is heterogeneous in its histopathology and sensitivity to chemotherapy. In order to overcome redundancies in the pathways and networks that control tumor cell growth, it will be necessary to employ multitargeted therapeutic strategies. A number of peritoneal structures could serve as useful potential targets, including inflammatory and non-inflammatory stromal cells, as well as production of molecules in the ECM, such as chemokines [29]. *In vitro* experiments suggest that the microenvironment can influence the malignant phenotype. It is also likely that malignant cells from the primary tumor or metastasis might modify the microenvironment, preparing both surface epithelial and stromal cells to support the growth and proliferative activity of the tumor. Thus, future strategies should attempt to identify those pathways and networks in the microenvironment that are critical to tumor cell survival.

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Inflammation

A hidden path to breaking the spell of ovarian cancer

Weiwei Shan and Jinsong Liu*

Department of Pathology; The University of Texas M.D. Anderson Cancer Center; Houston, TX USA

Key words: inflammation, epithelial ovarian cancer, fallopian tube, tumor microenvironment, cellular senescence

Abbreviations: BRCA, breast cancer; CCL2, chemokine (C-C motif) ligand 2; CCL5, chemokine (C-C motif) ligand 5; EOC, epithelial ovarian cancer; Gro-1, growth-regulated oncogene; ICAM1, intercellular adhesion molecule 1; IGFbps, insulin-like growth factor binding proteins; ILs, interleukins; MCP-1, monocyte chemotactic protein 1; MMPs, matrix metalloproteinases; OSE, ovarian surface epithelium; RANTES, regulated upon activation, normally T-expressed, and presumably secreted; SASP, senescence-associated secretory phenotype; TIMPs, tissue inhibitor of metalloproteinases; uPAR, urokinase plasminogen activator receptor; VCAM1, vascular cell adhesion molecule 1

Epithelial ovarian cancer is a highly lethal gynecological cancer for which overall prognosis has remained poor over the past few decades. A number of theories have been postulated in an effort to explain the etiology of epithelial ovarian cancer, each of which has been both applauded and doubted. Of note, these theories likely are not mutually exclusive, as they all converge more or less on the role of inflammation in promoting ovarian tumorigenesis. In this review, we describe the latest studies on the role of inflammation in the initiation and progression of epithelial ovarian cancer from three major aspects: physiological functions of a normal ovary, potential involvement of the fallopian tube in the initiation of epithelial ovarian cancer and the strong impact of the cellular microenvironment on the development of the disease.

Introduction

Epithelial ovarian cancer (EOC), the most common subgroup of ovarian cancer, is the deadliest gynecological cancer in the United States, accounting for more deaths than all other gynecological cancers combined.¹ The high mortality rate for EOC is a result of technical obstacles to early detection of the disease and a high prevalence of distal metastasis at late stages of the disease [(70% of cases)²]. This latter property is probably attributable to the unique peritoneal environment of EOC, which facilitates convenient seeding of ovarian cancer cells in the peritoneal cavity, which is further aided by the constant flow of peritoneal fluid.³ We call particular attention to this “open” environment to which EOC is exposed, because it has resulted in a myriad of characteristics specific to EOC, such as ease of widespread cancer metastases in a short period of time, unique formation of ascites, and high susceptibility of the ovarian surface epithelium (OSE) to peritoneal inflammatory stimuli.

Etiology of EOC: Inflammatory in Nature

EOC is perhaps one of the most sinuous human cancers. In an effort to identify the causes of EOC, a few hypotheses have been put forward. Two of these theories—the incessant ovulation hypothesis and the gonadotrophin hypothesis—are the most dominant in the ovarian cancer society. Proposed in the early 1970s, the incessant ovulation hypothesis attributes the formation of EOC to continuous damage and repair of the ovarian surface epithelia during cyclical ovulatory processes, which increase the chances for replicative DNA errors to be incorporated in ovarian epithelial cells.⁴ The gonadotrophin hypothesis, on the other hand, suggests that excessive exposure of the ovarian surface epithelia to gonadotrophins can result in enhanced epithelial cell proliferation and malignant transformation.⁵ A third theory emerged in the late 1990s which states that hormonal influences, including androgen and progesterone, have a major impact on the proliferation of the ovarian surface epithelia and, hence, EOC.⁶

Unlike that of the majority of other organs, the surface epithelium of the ovary is a natural continuant of the peritoneal lining and thus is directly exposed to any metabolic, environmental and xenobiotic stress present in the peritoneal cavity, most of which have inflammatory properties. However, the sources of inflammatory stimuli to which the ovary is exposed remain under-characterized. In fact, more than a decade ago, the primary physiological function of the ovary, ovulation, was found to be pro-inflammatory in nature⁷ and potentially mutagenic.⁸ As we focus on the molecular events that take place in the pre-ovulatory ovary, we will find that as the pre-ovulatory follicle matures, the proximal ovarian epithelial cells proliferate⁹ and then undergo apoptosis¹⁰ to accommodate follicular growth. Meanwhile, the fibroblastic layers of the tunica albuginea and theca externa are weakened in preparation for ovulation. These events culminate in a burst of apical epithelial cells and the underlying follicular layers followed by rapid extrusion of the ovum, wounding the surface of the ovary.¹¹ Intriguingly, these ovulatory processes, together with the repair steps immediately after liberation of the ovum, are marked by generation of an enormous body of

*Correspondence to: Jinsong Liu; Email: jliu@mdanderson.org
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cytokines/chemokines and matrix-remodeling enzymes, including prostaglandins, bioactive eicosanoids, plasminogen activators, collagenases, interleukins (ILs), tumor necrosis factors and various growth factors,^{12,13} as well as by recruitment of activated immune cells to the wounded epithelial surface, implying the occurrence of global activation of the pro-inflammatory network. Thus, the strong inflammatory stimuli, being both triggers and natural by-products of ovulation, may cause additional damage to the ovarian surface epithelia, which is already under tremendous stress because of the ovulatory rupture of the local epithelial cell layer. Not surprisingly, this panel of inflammatory modulators activated during cyclical ovulation has been found to exhibit a striking overlap with that described for EOC, including IL-8, CCL2/MCP-1 and CCL5/RANTES.¹⁴ Therefore, the incessant ovulation hypothesis has, perhaps inadvertently, provided evidence that inflammatory responses induced under physiological conditions may foster the development of EOC. Similarly, studies have shown that elevation of estrogens^{15,16} and androgens,¹⁷ as proposed by the gonadotrophin hypothesis and hormonal hypothesis, respectively, amplifies immune responses by recruiting pro-inflammatory cells and molecular effectors. Collectively, hypotheses attributing EOC to ovulation, gonadotrophin release, and hormonal influences likely are not mutually exclusive and lend strength to suggest that normal physiological activities of the ovary are accompanied by general activation of inflammatory mediators, which may either directly cause EOC or gradually produce genomic damage to the ovarian surface epithelia, until a future bolus dose of pathological stress brings the overall mutational tally above the threshold of ovarian tumorigenesis.

Inflammation, Tubal Tumorigenesis and Ovarian Cancer

Although the conventional view regarding ovarian cancer development is that more than 90% of cases originate from the OSE, the latest evidence points to hypothetical involvement of the fallopian tube, in particular, the fimbriated end of the tube, in the formation of serous ovarian cancer, prompted by findings presented by Crum et al. and other investigators. These authors demonstrated that examination of fallopian tubes and ovaries taken from BRCA-mutant women undergoing prophylactic salpingo-oophorectomy identified precursor lesions of serous ovarian carcinomas, unexpectedly, only in the tubal fimbria, not in the ovary.¹⁸⁻²⁰ Thus, at least in some cases, the fimbriated end of the fallopian tube may be the culprit in seeding of serous ovarian cancer.

Inflammatory insults to the fallopian tube can lead to tubal epithelial carcinogenesis. For instance, luminal dilatation and plical atrophy in the fallopian tube caused by chronic infection has been documented in many cases of primary fallopian tubal carcinomas.²¹ Exposure of the fallopian tube to inflammatory insults may occur physiologically and pathologically. Under physiological conditions, the retrograde flow of endometrial fluid during menstruation renders the fallopian tube acutely inflammatory by exposing the tube to a plethora of inflammatory molecules, including IL-8, tumor necrosis factor- α , and granulocyte-macrophage

colony-stimulating factor, all of which have been shown to be elevated in ovarian tumor specimens.²²⁻²⁴ Furthermore, the functional tubal fimbria, which has two epithelial surfaces—ciliated epithelium (endosalpinx) and peritoneal mesothelium—may be an area of continuous abrasion, stress-induced inflammation, and consequently, a site of cancer initiation.²⁵ Examples of endosalpinx-peritoneal junction-associated cancers include cervical²⁶ and gastroesophageal²⁷ malignancies, where the cervical squamous columnar junction and esophagogastric junction are located, respectively. In comparison, pathological inflammatory agents, including those that travel up from the lower female genital tract to the fallopian tube, are found frequently and to blame for a large proportion of female infertility. For example, pelvic exposure to asbestos²⁸ and to the sexually transmitted pathogen *Chlamydia trachomatis*²⁹ is known to cause tubal inflammation, also known as salpingitis. Taken together, these findings indicate that tubal inflammation is common under both pathological and non-pathological conditions.

Because inflammation is known to be a causal factor in promoting tubal tumorigenesis, the hypothesis that a portion of serous ovarian carcinomas may originate in the fallopian tube provides another link, although indirect, between inflammation and EOC. Recent studies, albeit preliminary, have associated inflammation of the fallopian tube with ovarian tumorigenesis, and supported studies indicating that the fallopian tube could be one of the origins of EOC. For example, the presence of chronic salpingitis has been found in 53% of ovarian carcinoma cases,³⁰ suggesting a causative relationship between the two. This notion is best supported by findings showing that hysterectomy and tubal ligation, both of which cut off the passage of inflammatory factors from the lower to the upper genital tract, afforded protection against EOC.³¹ More importantly, hysterectomy alone without tubal ligation was less effective in protecting against EOC than was hysterectomy combined with tubal ligation,³² emphasizing the significance of the fallopian tube in initiation of EOC. Although the hypothesis that (some of) serous ovarian cancers may stem from the tubal fimbria is still heatedly debated and calls for more substantial evidence, it for another time, perhaps unintentionally, supports the hypothesis that ovarian cancer is by nature inflammatory.

Inflammation, Cellular Senescence in the Ovarian Epithelial Microenvironment and Ovarian Cancer

As described above, the complex biology of the OSE makes ovarian epithelial cells exceedingly sensitive to peritoneal inflammatory agents. However, this is only half the story; the other half resides in the cellular microenvironment created by the ovarian stromal cells, in particular, aged or senescent stromal cells. Cellular senescence was initially described as an evolutionary advantage endowing cancer prevention when the cells entered an irreversible status of cell cycle arrest³³ in response to a variety of internal and external stimuli.^{34,35} In contrast with the conventional view that senescence is inherently protective against cancer, mounting evidence points to an unexpected role of senescent stromal cells, mainly stromal fibroblasts, in enhancing epithelial

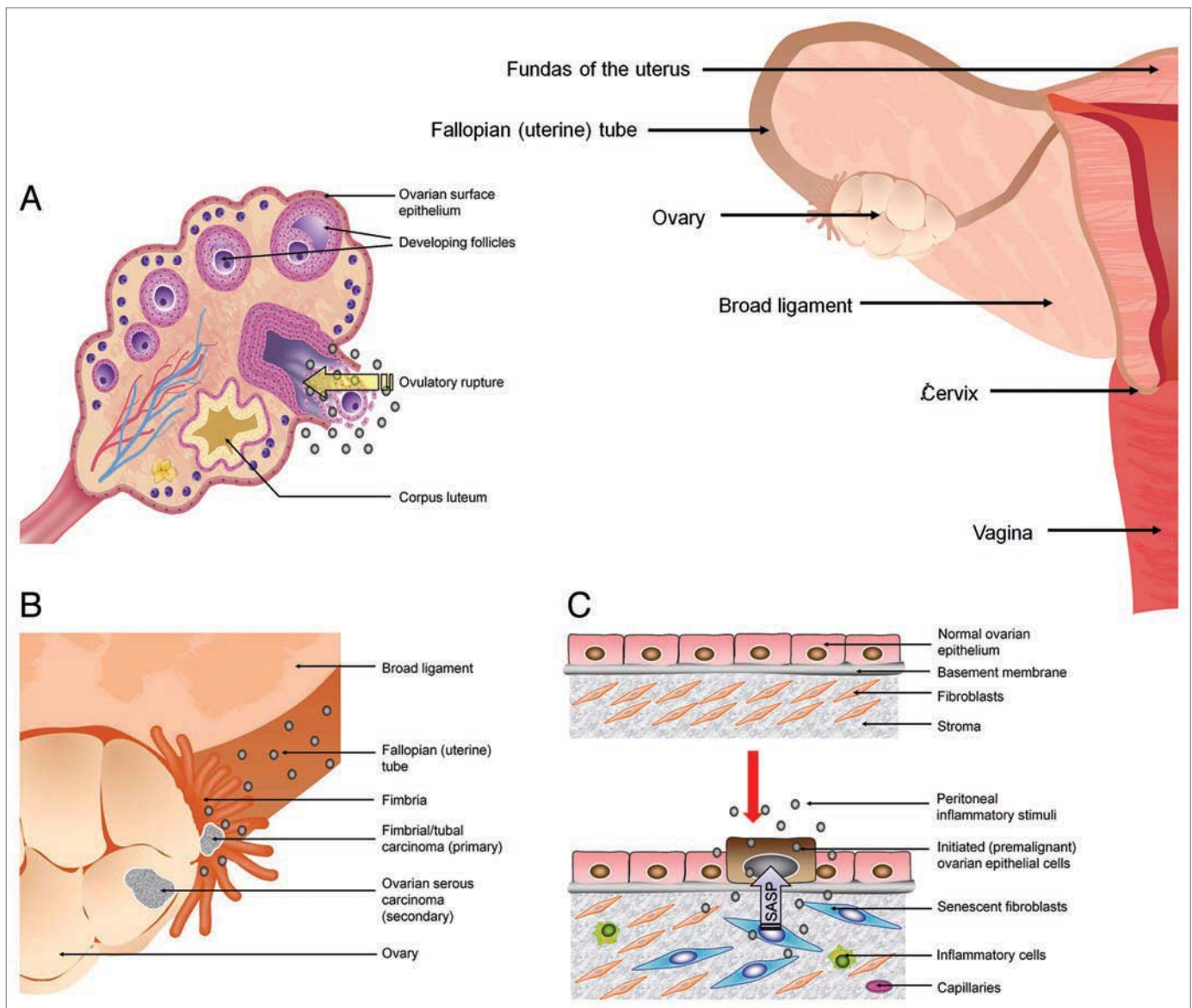


Figure 1. Potential sources of inflammatory stimuli that may contribute to the initiation and/or progression of EOC. A schematic representation of the left half of the female reproductive organs is shown at top right. (A) Normal functions of the ovary, such as ovulation, may be pro-inflammatory in nature. (B) Inflammatory insults to the fallopian tube can indirectly damage the adjacent ovarian surface epithelium. (C) Inflammatory molecules present in the peritoneal cavity may not only be mutagenic to the ovarian surface epithelium but also render ovarian stromal fibroblasts senescent. Subsequently, senescent fibroblasts create a secondary hyper-inflammatory microenvironment (SASP), together with inflammatory mediators in the peritoneal macroenvironment, contributing to the enhancement of EOC.

tumorigenesis. Specifically, Krtolica and colleagues showed that senescent but not normal human fibroblasts were markedly tumorigenic in premalignant (initiated but non-tumorigenic) human skin epithelial cells both in culture and in immune-compromised mice,³⁶ thus providing experimental proof that senescent stromal fibroblasts can augment epithelial tumorigenesis. Therefore, cellular senescence acts as a double-edged sword by either dampening or boosting tumorigenesis depending on the specific cell type and combination of intracellular and extracellular factors. Accumulating evidence has suggested that diffusible paracrine signaling molecules secreted by senescent fibroblasts orchestrate the senescence-associated enhancement of tumorigenesis by

fine-tuning the epithelial microenvironment into one favorable for tumor growth. Thus far, a broad spectrum of pro-inflammatory mediators have been reported to be markedly activated in senescent cells, including myriad ILs (e.g., IL-6, IL-8, IL-1 β), chemoattractants (e.g., Gro-1/ α , MCP-1, CSMF), matrix-remodeling enzymes (e.g., MMPs, TIMPs, uPAR), and adhesion molecules (e.g., ICAM-1, VCAM-1, integrins),³⁷ suggesting that upon senescence, aged cells take up the highly pro-inflammatory “senescence-associated secretory phenotype” (SASP).³⁸

Our laboratory has found direct evidence that senescent ovarian fibroblasts promote ovarian epithelial tumorigenesis by mobilizing the pro-inflammatory network. Recently, we demonstrated

that expression of the chemokine Gro-1/ α was induced in HRAS^{V12}-transformed ovarian epithelial cells and that epithelial cell-released Gro-1/ α mediated the senescence of ovarian stromal fibroblasts by diffusing into the stroma and acting non-autonomously on fibroblasts.³⁹ Subsequently, ovarian fibroblasts rendered senescent by Gro-1/ α proved to be tumor-promoting of initiated ovarian epithelial cells when co-injected into nude mice with the latter,³⁹ which was consistent with results reported previously by others. In addition to Gro-1/ α , we have also observed elevated expression of a wide spectrum of pro-inflammatory cytokines and chemokines in HRAS^{V12}-transformed ovarian epithelial cells than in their immortalized, non-tumorigenic parental cells.⁴⁰ When this panel of RAS-induced secreted factors was compared with the SASP described in Coppe's study,³⁸ a considerable overlap between these two was identified, including IL-6, IL-8, Gro-1/ α , Gro-2/ β , ICAM-1, IGFBP-1 and MCP-1 (reviewed in refs. 40 and 41 and unpublished data from us). Some of these factors are established senescence inducers,^{39,42-44} suggesting that many, if not all, of the HRAS^{V12}-induced inflammatory molecules could also mediate cellular senescence. Do senescent stromal fibroblasts enhance human EOC in vivo? The answer to this question is probably yes. We have detected senescent ovarian stromal fibroblasts adjacent to human ovarian tumor epithelium in clinical specimens,³⁹ supporting the existence of such cells in human cases of ovarian cancer. Although evidence supporting a senescence-associated pro-inflammatory secretome acting in a paracrine fashion on ovarian tumor epithelium in vivo has been lacking, postulating that inflammation-mediated stromal senescence can play a critical role in triggering as well as promoting human EOC is reasonable. Collectively, we have shown that in oncogenic RAS-transformed ovarian epithelial cells, a drastically pro-inflammatory secretome is generated, which can diffuse into the stroma and cause senescence in stromal fibroblasts. Conversely, senescence of ovarian stromal fibroblasts may contribute to progression of EOC by creating a secondary pro-inflammatory phenotype (SASP) and converting the ovarian epithelial microenvironment into one filled with inflammatory mediators

that favor tumor advancement. The central role of the inflammatory network in interweaving these events is prominent, which directs extensive cellular communications between the ovarian tumor epithelium and the underlying stroma that converge on the augmentation of EOC.

Conclusions

The tumor milieu in which EOC develops has been described as one enriched with a broad spectrum of pro-inflammatory cytokines and chemokines.¹⁴ Increasing evidence suggests that inflammation contributes significantly to the etiology of EOC. Studies have not only shown that physiological ovarian functions are pro-inflammatory in nature (Fig. 1A) but also suggested that activities that take place in the fallopian tubes influence EOC (Fig. 1B). More recent studies of cellular senescence have revealed a potential role for senescent stromal fibroblasts in the augmentation of EOC by increasing the expression of diffusible inflammatory mediators (Fig. 1C). Intriguingly, cellular senescence, which itself serves as a vast repertoire of inflammatory molecules, can be induced by physiologically and pathologically derived inflammatory agents in the peritoneal macroenvironment and/or cellular microenvironment; when these stimuli work in synergy, the ovarian epithelial tumor milieu becomes exponentially inflammatory and favorable for cancer development. A more comprehensive understanding of these issues will benefit the cancer pharmaceutical industry in designing new strategies for the treatment and prevention of human EOC.

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Exhibit 110



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Pre-diagnostic serum levels of inflammation markers and risk of ovarian cancer in the Prostate, Lung, Colorectal and Ovarian Cancer (PLCO) Screening Trial

Britton Trabert¹, Ligia Pinto^{1,2}, Patricia Hartge¹, Troy Kemp², Amanda Black¹, Mark E. Sherman³, Louise A. Brinton¹, Ruth M. Pfeiffer¹, Meredith S. Shiels¹, Anil K. Chaturvedi¹, Allan Hildesheim¹, and Nicolas Wentzensen¹

¹Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA

²HPV Immunology Laboratory, Frederick National Laboratory for Cancer Research, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Frederick, MD, USA

³Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Department of Health, and Human Services, Bethesda, MD, USA

Abstract

Objective—Pro-inflammatory mechanisms may explain the increased ovarian cancer risk linked to more lifetime ovulations, endometriosis, and exposure to talc and asbestos, as well as decreased risk with non-steroidal antiinflammatory drugs. Limited data are available to estimate ovarian cancer risk associated with levels of circulating inflammatory markers.

Methods—We conducted a nested case-control study within the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. Pre-diagnostic serum levels of 46 inflammation-related biomarkers (11 with *a priori* hypotheses; 35 agnostic) were measured in 149 incident ovarian cancer cases and 149 matched controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using conditional logistic regression and adjusted for identified covariates.

Results—Increased ovarian cancer risk was associated with elevated levels of C-reactive protein (CRP) [tertile (T)3 vs. T1: OR (95% CI) 2.04 (1.06-3.93), p-trend=0.03], interleukin (IL)-1 α [detectable vs. undetectable: 2.23 (1.14-4.34)] and tumor necrosis factor alpha (TNF- α) [T3 vs. T1: 2.21 (1.06-4.63), p-trend=0.04] Elevated IL-8 was non-significantly associated with risk [T3 vs. T1: 1.86 (0.96-3.61), p-trend=0.05] In analyses restricted to serous ovarian cancer (n=83), the associations with CRP and IL-8 remained or strengthened [CRP T3 vs. T1: 3.96 (1.14-11.14), p-trend=0.008; IL-8 T3 vs. T1: 3.05 (1.09-8.51), p-trend=0.03]. Elevated levels of CRP and TNF- α

Corresponding author: Britton Trabert, 9609 Medical Center Drive, Room 7E-228, Bethesda, MD 20892-9774, Phone: 240-276-7331, Fax: 240-276-7838, britton.trabert@nih.gov.

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remained positively associated with ovarian cancer risk in analysis restricted to specimens collected at least 5 years before diagnosis (n=56).

Conclusion—These results suggest that CRP, IL-1 α , IL-8, and TNF- α are associated with increased risk of subsequently developing ovarian cancer.

Introduction

Epidemiologic evidence implicates chronic inflammation as a central mechanism in the pathogenesis of ovarian cancer, the most lethal gynecologic cancer among women in the United States.[1] Chronic inflammation can induce rapid cell division, increasing the possibility for replication error, ineffective DNA repair and subsequent mutation. Ovarian cancer has been linked to several events and conditions which are related to inflammation and repair, including incessant ovulation, endometriosis, exposure to talc and asbestos, and in some studies pelvic inflammatory disease.[Reviewed in [2]] In addition, reduced risks found for aspirin use [3] could be related to direct anti-inflammatory actions, while reduced risks related to tubal ligation and hysterectomy could reflect limited exposure to environmental causes of inflammation.[2] Understanding the role of inflammation in ovarian cancer etiology is complicated by growing recognition that there are at least two main types of these tumors, which differ clinically and biologically.[4] Increasing evidence suggests that some high-grade serous carcinomas, the most common and lethal subtype, arise from the fimbria of the fallopian tube rather than the ovarian surface epithelium.[4]

Recent clinical and prospective data suggest that C-reactive protein (CRP), a marker of global inflammation, is associated with increased ovarian cancer risk.[5-8] Pre-diagnostic CRP levels have been associated with ovarian cancer risk in all four studies[5-8] evaluating the association; with one study showing an association only among women with “clinically high” CRP levels (>10 mg/L vs. <1 mg/L).[6]

Other inflammatory markers may be important in ovarian carcinogenesis. In premenopausal women ovarian epithelial cells secrete cytokines as part of ovarian function and some of these cytokines are also produced by ovarian cancer cells.[9-11] Follicle rupture during ovulation involves tissue remodeling with high cell turnover that is characteristic of inflammatory reactions. Many inflammatory mediators, including prostaglandins, leukotrienes, and cytokines, are locally elevated during ovulation.[12] Epithelial cells in proximity to ovulating follicles are likely exposed to these inflammatory mediators that may signal oxidative stress, and enhance the risk of mutagenesis. In addition, data from animal and limited human studies supports the hypothesis that ovulation may trigger cellular events that result in carcinogenesis.[13, 14] Importantly, cytokines involved in ovarian function, follicle rupture, and repair (physiologic processes before menopause) are suggested to remain activated in postmenopausal women and may play an etiologic role in ovarian carcinogenesis; these cytokines include: interleukin (IL)-1 α , IL-1 β , IL-2, IL-6, IL-8, IL-10, tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF).[11]

To gain a better understanding of the etiologic role of inflammation markers in ovarian cancer development, we conducted a nested case-control study within the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. We used multiplexed inflammatory marker panels to measure 46 inflammation-related markers, including several inflammation markers with existing evidence of associations with ovarian function or ovarian cancer risk.

Materials and Methods

Study Design

We conducted a nested case-control study within the screening arm of the PLCO Cancer Screening Trial. Details of the screening trial have been reported previously.[15] Briefly, between 1993 and 2001, approximately 155,000 subjects (78,216 women) 55-74 years of age were recruited from ten cities from the general population and randomized to the screening or non-screening arm of the study. Screening-arm subjects provided blood samples at baseline and five subsequent annual medical examinations. Samples were processed and frozen within two hours of collection, and stored at -70 degrees Celsius.[16] In addition to trial cancer outcomes (prostate, lung, colorectal and ovarian cancers) detected by annual screening examinations during the first six years of follow-up, individuals were followed for all cancer diagnoses by annual mailed questionnaires. All cancer diagnoses were pathologically confirmed through medical record abstraction. Institutional review boards of the U.S. National Cancer Institute and the ten study centers approved the trial, and all participants provided written informed consent. The nested case-control study was also approved by the institutional review board of the National Cancer Institute.

We identified 150 first-primary ovarian cancer cases diagnosed between two and fourteen years after blood collection from among the eligible screening-arm participants followed through December 31, 2008. Eligibility criteria included the availability of an unfrozen serum sample, consent to biochemical studies, completion of the baseline questionnaire, and no history of cancer (other than non-melanoma skin cancer) prior to ovarian cancer diagnosis. Serum specimens from a single visit were measured for each study subject. To ensure a relatively equal distribution of specimens between 2 and 14 years prior to diagnosis, 11.4% of samples selected were measured at baseline and the remaining at follow-up visits (18.1% year 1, 26.2% year 2, 12.8% year 4, and 31.5% year 5). Controls were individually matched to cases on the basis of age at blood collection (55-59, 60-64, 65-69, 70+ years), race (white, black, other), study center, and time (a.m., p.m.) and date (three-month categories) of blood collection. Controls were restricted to women with no history of oophorectomy at the time of diagnosis of their matched case. We were unable to identify a suitable matched control for one case, therefore our final analytic sample consisted of 149 cases and 149 matched controls.

Laboratory Methods

We measured circulating levels of 60 immune and inflammation markers, including cytokines, chemokines, growth factors, and soluble products of immune activation (Supplemental Table 1). Assays for these markers have demonstrated satisfactory performance and reproducibility [17] and include assessment of 11 markers linked with

either ovulation or ovarian cancer risk. Fifty-nine of the 60 markers were measured on four Luminex bead-based commercial assay panels (Millipore Inc., Billerica, MA). The remaining marker, CRP, was measured with a Luminex bead-based assay from Millipore (Billerica, MA) and tested according to the manufacturer's protocol. Batched assays were performed in a single laboratory (LP). Concentrations of the 60 multiplexed markers were calculated using a four- or five-parameter logistic curve using Bioplex Manager 6.1 software (BioRad, Hercules, CA). Cases and matched controls were included in the same analytic batch. Samples were assayed in duplicate and averaged to calculate concentrations. To evaluate assay performance we included a replicate sample from a quality control (QC) pool in each batch. Percent detected above the lower limit of detection (LLOD), coefficients of variation (CVs), and intraclass correlation coefficients (ICCs) for the QC samples of all measured inflammation markers are summarized in Supplemental Table 1. We excluded from further study 14 markers with <20% of values above the LLOD. Although IL-1 α had only 18.4% of values above the LLOD we included this marker in analyses because it was one of eleven markers with *a priori* hypothesis regarding a potential ovarian cancer association and it was close to the 20% threshold. After these exclusions, 46 markers were included in the statistical analysis.

Statistical Analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between the serum inflammation markers and ovarian cancer risk were calculated using conditional logistic regression models. All models were adjusted for *a priori* potential confounding factors parity (nulliparous, parous), duration of oral contraceptive use (never, 1-5 years, 6+ years), duration of menopausal hormone therapy use (never, 1-5 years, 6+ years), cigarette smoking status (never, former, current) and body mass index (BMI; <25, 25-29.9, 30+ kg/m²). Further adjustment by aspirin or ibuprofen use, or family history of breast or ovarian cancer, did not substantially change the observed effect estimates, therefore we did not include these covariates in the model. Marker levels were categorized into groups based on the proportion of individuals with measurements above the LLOD as follows: markers with 66% of individuals with measurements above LLOD or greater (n=26) were categorized into tertiles based on the distribution among controls, individuals with values at or below LLOD were included in the lowest tertile; markers with fewer than 66% of individuals with measurements above LLOD were categorized into two groups (detectable vs. non-detectable (\leq LLOD)). To compute tests for trend across tertile categories, intracategory medians were modeled as a continuous parameter. Q-values which reflect the false discovery rate (FDR) were calculated to account for multiple comparisons.

In secondary analyses, we evaluated associations stratified by serous/non-serous histologic subtype as well as time between blood collection and diagnosis (2-<5 years and 5-14 years). Given the modest correlation between the markers, we further evaluated those markers that were associated with ovarian cancer risk in a mutually adjusted model. For the analysis of CRP, we conducted a sensitivity analysis excluding individuals who reported current use of menopausal hormone therapy at blood draw, as a high CRP level in women taking hormone therapy may be due to a first pass effect.[18] We also conducted a sensitivity analysis excluding individuals with known inflammatory conditions: cardiovascular disease,

rheumatoid arthritis, and diabetes (n=146). We further examined associations modeling the cross-classification of the inflammatory marker and its modulator (e.g. TNF- α and its receptor TNF- α -R1). Finally, given that cancer antigen (CA)-125 is currently the best predictor of ovarian cancer we evaluated the correlation between CA-125 and the inflammatory marker level from the same study year. Correlation coefficients for the markers evaluated were non-significant and less than 0.15 (results not shown). Further, only 5 subjects were classified as CA-125 positive at the corresponding study year of blood draw, therefore further model adjustment for CA-125 was uninformative.

Results

The distribution of selected demographic and health characteristics of the cases and controls are summarized in Table 1. Participants were on average 63 years old at enrollment and were predominately white (92.6%). The median length of follow-up from blood collection to case diagnosis was 4.2 years (interquartile range (IQR): 2.8-6.7 years). The median length of follow-up from blood collection until the end of follow-up for controls was 9.9 years (IQR: 8.0-12.9).

Of the eleven markers with an *a priori* hypothesis regarding a potential ovarian cancer association (CRP, IL-1 α , IL-1 β , IL-2, IL-6, IL-8, IL-10, TNF- α , IFN- γ , G-CSF, and GM-CSF), four were positively associated with ovarian cancer risk in the current study (Table 2): CRP [tertile (T)3 vs. T1: OR (95% CI) 2.04 (1.06-3.93), p-trend=0.03], IL-1 α [detectable vs. undetectable: 2.23 (1.14-4.34)], TNF- α [T2 vs. T1: 1.89 (1.01-3.53), T3 vs. T1: 2.21 (1.06-4.63), p-trend=0.04] and IL-8 [T3 vs. T1: OR 95% CI 1.86 (0.96-3.61), p-trend=0.05]. The association with IL-1 α is based on 34 exposed cases only and should be interpreted with caution. In analyses restricted to serous ovarian tumors (n=83), the associations with CRP, IL-1 α , and IL-8 remained [CRP T3 vs. T1: OR (95% CI) 3.96 (1.14-11.14), p-trend=0.008; IL-1 α detectable vs. non-detectable: OR (95% CI) 2.70 (1.10-6.36); IL-8 T3 vs. T1: OR (95% CI) 3.05 (1.09-8.51), p-trend=0.03] (Table 3). The association for serous tumors with TNF- α was no longer statistically significant [T3 vs. T1: OR 95% CI 2.06 (0.71-6.00), p-trend=0.19]; however TNF- α was associated with an increased risk in analyses restricted to non-serous ovarian tumors (n=76) [T2 vs. T1: 4.92 (1.52-15.90), T3 vs. T1: 4.36 (1.11-17.05), p-trend=0.05]. After correction for multiple comparisons, CRP was significantly associated with serous ovarian cancer at FDR less than 0.10. The q-values for the associations between CRP, IL-1 α , TNF- α , IL-8 and ovarian cancer risk were 0.13. The q-values for the remaining associations in Tables 2 and 3 were all greater than 0.13.

Of the remaining 35 markers with weak or no prior evidence of an association (Supplemental Tables 2 and 3), three were positively associated with ovarian cancer risk. Among the markers with 66% of individuals with measurements above the LLOD (Supplemental Table 2), interferon gamma-induced protein 10 (IP-10) and macrophage inflammatory protein-1 β (MIP-1 β) were associated with increased ovarian cancer risk comparing the second tertile to the first tertile; however, the trend across tertiles and the association comparing the third tertile to the first tertile were not statistically significant. Among markers with fewer than 66% of individuals with measurements above the LLOD (Supplemental Table 3), fibroblast growth factor 2 (FGF-2) was associated with increased

risk [detectable vs. \leq LLOD FGF-2: OR (95% CI) 2.21 (1.15-4.25)]; however, this result should be interpreted with caution, given it is based on 28 exposed cases. The remaining markers evaluated were not associated with increased or decreased ovarian cancer risk (Supplemental Tables 2 and 3, and Figure 1). The q-values for all markers evaluated in Supplemental Tables 2 and 3 were all > 0.10 .

In analyses restricted to cases with specimens collected at least five years prior to diagnosis (n=56), CRP and TNF- α levels remained positively associated with ovarian cancer risk [CRP T3 vs. T1: OR (95% CI) 4.51 (1.08-18.82), p-trend=0.03; TNF- α T3 vs. T1: OR (95% CI) 5.55 (1.19-25.83), p-trend=0.04] (results not tabled), while the trend across tertiles for IL-8 was no longer statistically significant [T3 vs. T1: OR (95% CI) 1.70 (0.55-5.27), p-trend=0.34] (results not tabled). Increased risk of ovarian cancer with IP-10 and FGF-2 remained in analyses restricted to specimens collected at least five years prior to diagnosis (results not shown).

In mutually adjusted models there was an independent association between CRP and ovarian cancer risk in the analysis of all cases (Table 4). In analyses restricted to serous tumors the increased risk with elevated serum levels of IL-8 and CRP remained in the mutually adjusted model, whereas in the analysis of specimens collected at least five years prior to cancer diagnosis both CRP and TNF- α were independently associated with increased risk. In contrast, in analyses restricted to specimens collected less than 5 years prior to diagnosis, ORs from the mutually adjusted model were not significantly elevated for CRP, IL-8 or TNF- α . Further, the increased risk of ovarian cancer with elevated CRP was not attenuated in an analysis restricted to women who did not report menopausal hormone use at the time of blood draw [OR T3 vs. T1= 2.21] (results not tabled). Results were not substantially attenuated after excluding cases and controls with cardiovascular disease, rheumatoid arthritis, and diabetes (results not shown). Finally, there were no statistically significant associations based on analyses modeling the cross-classification of the inflammatory marker and its modulator (results not shown).

Discussion

We identified several circulating inflammation markers that were associated with risk of developing ovarian cancer between 2 and 14 years later. Specifically we observed associations between elevated CRP, IL-1 α , IL-8, and TNF- α and risk of epithelial ovarian cancer in a nested case-control study in the PLCO Cancer Screening Trial. For CRP and TNF- α , we found the same effects for serum samples collected 5 or more years prior to diagnosis, supporting that reverse causation does not explain the effect.

Data from animal and limited human studies support the hypothesis that ovulation may trigger cellular events that result in carcinogenesis. Hyperovulatory hens have markedly increased likelihood of developing ovarian adenocarcinomas, as do rats with hyper-proliferating ovarian epithelial cells [13, 14]. It is plausible that cytokines play a role in the development of pre-neoplastic cells in the epithelium that, under continuous cytokine stimulation, progress to cancer cells, suggesting that elevated levels of these cytokines may confer increased ovarian cancer risk [9-11]. Further, it has been shown that ovarian

epithelial cells secrete cytokines and that these same factors are also produced by ovarian cancer cells further supporting that the recruitment of normally secreted cytokines into dysregulated autocrine loops may be important in neoplastic progression.[9, 10]

Our results further support the association between pre-diagnostic CRP levels and ovarian cancer observed in four previous studies.[5-8] CRP is a marker of global inflammation that has been associated with other cancers. It is not clear whether CRP directly influences ovarian carcinogenesis or is an indirect marker of inflammatory exposures to the ovary. One study suggested that high levels of CRP in ovarian cancer patients was correlated with an impaired T-cell response [19] and several small studies generally observed that circulating or peritoneal CRP levels were higher during post-ovulatory phases of the menstrual cycle, [20-24] indicating that CRP may be involved in the local wound healing process following ovulation. CRP remained the dominant risk factor as the associations for IL-8 and TNF- α were attenuated after mutual adjustment for CRP.

Our study is the first to show an association between elevated circulating IL-1 α and ovarian cancer risk; however, given that only 18.4% of values were above the LLOD for this marker, these results should be interpreted with caution. IL-1 α is produced following nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation,[25] and signaling of IL-1 α through its receptor results in downstream activation of NF- κ B,[26] which leads to transcription of a number of genes whose products promote inflammation. [27] This pathway appears to play a crucial role in the process that links inflammation to cancer.[28, 29] Specifically, activation of NF- κ B through inhibitor of κ B kinase epsilon (IKK ϵ) was shown to be associated with more aggressive behavior in ovarian cancer cell lines [30] and has been associated with aberrant cellular activities in endometriosis, a known risk factor for ovarian cancer.[31]

No previous study has shown an association between elevated circulating IL-8 and ovarian cancer using prediagnostic samples, the higher risk in serum samples collected in the most recent 5 years before diagnosis is consistent with evidence implicating the IL-8 pathway in later steps of carcinogenesis, including tumor progression and metastasis.[32] IL-8 has been shown to be elevated in ovarian cyst fluid, ascites, serum and tumor tissue from ovarian cancer patients and increased IL-8 expression correlates with poor prognosis and survival. [33-39]

TNF- α , like CRP, is a marker of various inflammation processes. TNF- α has been shown to play a role in later steps of carcinogenesis.[40, 41] For example, NF- κ B activation by TNF- α is involved in neoplastic transformation, proliferation, and tumor survival.[42] In addition, in ovarian cancer cells, TNF- α enhances cell migration and metastasis through NF- κ B-dependent induction of IL-8, C-X-C chemokine receptor type 4 (CXCR4), monocyte chemoattractant protein 1 (MCP-1), and intercellular adhesion molecule-1.[43] TNF- α was positively associated with ovarian cancer in case-control studies using serum samples collected at diagnosis.[19, 44] We report an increased risk of ovarian cancer with TNF- α measured in pre-diagnostic serum. Our finding is not consistent with the null association reported by Clendenen et al.,[45] however, the elevated, albeit not statistically significant,

OR for TNF- α -receptor 2 observed in our study was consistent with the increased ORs reported by Poole et al.[7]

Inconsistent results in the existing studies may reflect limited case numbers in cohort studies that collected prediagnostic specimens. Further, the use of different inflammation marker assays may have led to differing results across the studies. The multiplex assays utilized in the current study are comparable to those used by Clenenden et al.,[45] however, the assay performance was noticeably different. For most of the inflammatory markers measured in the two studies, the percent of markers below LLOD was higher in the current study. Specifically, the low percent detection limited the ability to evaluate some markers[46] that were associated with ovarian cancer (i.e. IL-6 and IL-12p40) in the study by Clenenden et al.[45] The assay performance in the current study was very similar to the systematic evaluation of multiplex inflammation marker panels published earlier by our group.[17]

The strengths of our study include the prospective design, comprehensive evaluation of inflammation-related markers measured using a validated technology, and careful control for confounding. We also note several limitations. Although we were able to include all ovarian cancer cases from the PLCO screening arm, the study was limited in power, which affected our ability to investigate associations with ovarian cancer subtypes other than serous tumors. Further, given the limited sample size, associations for all markers tested were imprecise. With respect to the inflammation hypothesis, however, the evidence is compelling for serous ovarian tumors, and many of the inflammation marker-ovarian cancer associations strengthened in these analyses. While our observations support the association of pre-diagnostic circulating markers of inflammation with ovarian cancer, they require replication given the large number of markers evaluated. Only the association with CRP and serous ovarian cancer was identified with an FDR less than 0.10. The associations between CRP, IL-1 α , IL-8, TNF- α and ovarian cancer had FDR q-values of 0.13, while the remaining markers evaluated were not associated with ovarian cancer risk after correction for multiple comparisons. Further, we measured markers at only one time point; however, data suggests that most of the markers are moderately stable over time, with ICCs of 0.54-0.67 for CRP over four years,[49, 50] and an ICC of 0.87 for TNF- α over three blood draws within two years.[51] In the only study published to date, the ICC for IL-8 was less stable (0.33 over two years).[51] It is important to note that several markers of inflammation, namely CRP and TNF- α , have also been associated with other tumors.[46-48] Presumably, these markers represent a common pathway of different inflammatory processes at different cancer sites. Future studies need to increase the focus on the tumor-specific inflammatory mechanisms that underlie the reported associations of systemic inflammation markers here and in other studies. Lastly we note that the circulating inflammation markers measured in the current study may not reflect levels in local sites of inflammation relevant to ovarian carcinogenesis, which may include the fallopian tube, ovary or endometriotic lesions. Studies investigating the correlation between serum inflammation marker levels and different tissue types, using animal or human clinical specimens, could provide important insight into this question. As mentioned, additional research is needed to confirm these findings and better understand the role that inflammation may play in the etiology of ovarian cancer. If confirmed, further evaluation of these markers in risk prediction models is warranted.

In conclusion, our prospective investigation of 46 inflammation-related markers provides evidence that serum levels of CRP and TNF- α are associated with increased future risk of ovarian cancer, 5 or more years following blood collection. We also observed ovarian cancer associations for several novel markers that warrant further investigation. Increased inflammation may be etiologically important in ovarian carcinogenesis arguing for additional research to confirm and extend these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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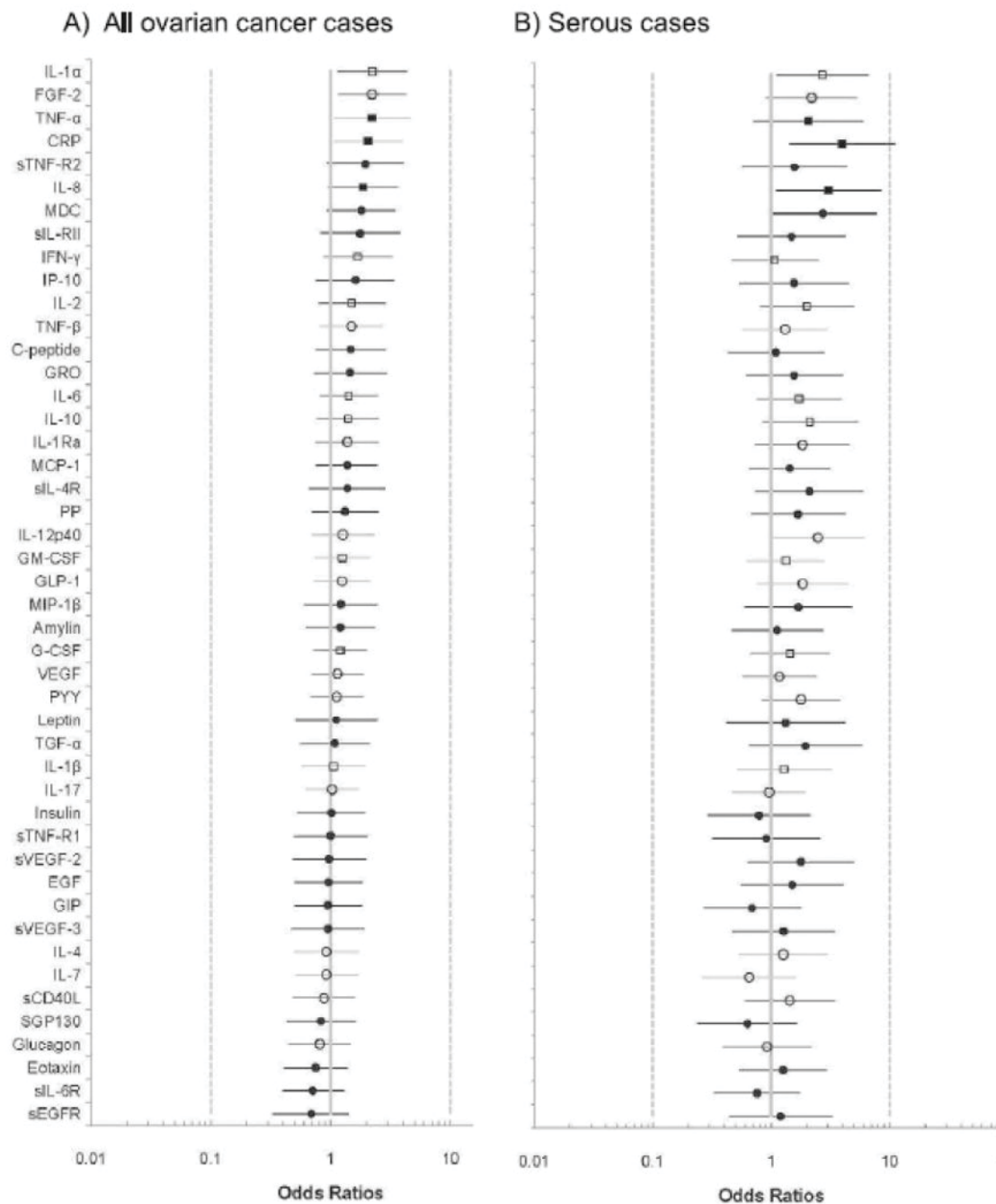
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Research Highlights

- We evaluated 46 pre-diagnostic inflammation-related biomarkers and ovarian cancer.
- CRP, TNF- α , and IL-8 are associated with increased risk of subsequently developing ovarian cancer.
- Increased risks with CRP and TNF- α are apparent 5 or more years prior to diagnosis.
- Our study provides additional evidence that inflammation plays an important role in ovarian carcinogenesis.

**Figure 1.**

Association between 46 inflammation markers and ovarian cancer risk using A) all ovarian cancer cases and B) serous ovarian cancer cases, nested case-control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. The symbol represents the odds ratio (OR) and the error bars represent the corresponding 95% confidence intervals. Filled markers indicate that the OR calculation was based on the comparison of individuals with marker measurements in Tertile 3 versus Tertile 1, unfilled markers indicate that the OR association is based on the comparison of individuals with marker measurements above

the lower limit of detection (LLOD) versus values at or below LLOD. Square symbols indicate the 11 markers with *a priori* hypothesis regarding an association with ovarian cancer risk and circle symbols indicate the remaining 35 markers with weak or no prior evidence for an association.

Table 1

Demographic and health characteristics of cases and controls, nested case-control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial.

	Cases (n=149)		Controls (n=149)	
	Mean	SD	Mean	SD
Age at baseline	63.2	5.5	63.0	5.3
Race	n ¹	%	n ¹	%
Non-Hispanic White	138	92.6	138	92.6
Non-Hispanic Black	5	3.4	5	3.4
Hispanic	3	2.0	3	2.0
Asian	3	2.0	3	2.0
Highest education level attained				
High school or less	39	26.2	47	31.5
Some post high school training	54	36.2	49	32.9
College graduate	56	37.6	53	35.6
Body Mass Index (kg/m2)				
< 25	65	43.6	64	43.0
25-29.9	54	36.2	53	35.6
≥ 30	28	18.8	32	21.5
Cigarette smoking status				
Never	80	53.7	95	63.8
Current	12	8.1	15	10.1
Former	57	38.3	39	26.2
Parity				
Nulliparous	10	6.7	5	3.4
Parous	139	93.3	144	96.6
Duration of oral contraceptive use				
Never	80	53.7	73	49.0
1-5 years	46	30.9	48	32.2
6+ years	23	15.4	28	18.8
Duration of menopausal hormone therapy use ²				
Never	39	26.2	59	39.6
1-5 years	48	32.2	47	31.5
6+ years	62	41.6	43	28.9

¹Values may not sum to total because of missing data.

²Frequency of duration of menopausal hormone therapy use was differed between cases and controls p-value<.05.

Table 2

Associations of *a priori* selected pre-diagnostic circulating inflammation markers and ovarian cancer risk, nested case-control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial.

	Cases (n=149)		Controls (n=149)		OR ¹	(95% CI)
	n	%	n	%		
C-reactive protein (CRP) (mg/L) < 3.23	39	26.2	49	32.9	1.00	(ref)
3.23-9.76	47	31.5	50	33.6	1.29	(0.68-2.41)
> 9.76	63	42.3	50	33.6	2.04	(1.06-3.93)
<i>p-trend</i>					0.03	
Interleukin (IL)-1α (ng/L) ² ≤ LLOD (3.2)	115	77.2	128	85.9	1.00	(ref)
Detectable	34	22.8	21	14.1	2.23	(1.14-4.34)
IL-1β (ng/L) ≤ LLOD (0.64)	110	73.8	111	74.5	1.00	(ref)
Detectable	39	26.2	38	25.5	1.06	(0.58-1.94)
IL-2 (ng/L) ≤ LLOD (0.64)	112	75.2	119	79.9	1.00	(ref)
Detectable	37	24.8	30	20.1	1.50	(0.79-2.84)
IL-6 (ng/L) ≤ LLOD (0.64)	100	67.1	108	72.5	1.00	(ref)
Detectable	49	32.9	41	27.5	1.41	(0.81-2.46)
IL-8 (ng/L) < 1.87	41	27.5	49	32.9	1.00	(ref)
1.87-3.79	43	28.9	50	33.6	1.17	(0.60-2.27)
> 3.79	65	43.6	50	33.6	1.86	(0.96-3.61)
<i>p-trend</i>					0.05	
IL-10 (ng/L) ≤ LLOD (0.64)	104	69.8	111	74.5	1.00	(ref)
Detectable	45	30.2	38	25.5	1.39	(0.77-2.50)
Interferon gamma (IFN-γ) (ng/L) ≤ LLOD (3.2)	116	77.9	119	79.9	1.00	(ref)
Detectable	33	22.2	30	20.1	1.68	(0.87-3.27)
Granulocyte colonystimulating factor (G-CSF) (ng/L) ≤ LLOD (16.0)	82	55.0	86	57.7	1.00	(ref)
Detectable	67	45.0	63	42.3	1.20	(0.72-2.01)
Granulocyte colony-stimulating factor (GM-CSF) (ng/L) ≤ LLOD (13.2)	86	57.7	91	61.1	1.00	(ref)
Detectable	63	42.3	58	38.9	1.25	(0.74-2.11)
Tumor necrosis factor alpha (TNF-α) (ng/L) < 4.05	36	24.2	49	32.9	1.00	(ref)

	Cases (n=149)		Controls (n=149)		OR ¹	(95% CI)
	n	%	n	%		
4.05-5.48	59	39.6	50	33.6	1.89	(1.01-3.53)
> 5.48	54	36.2	50	33.6	2.21	(1.06-4.63)
<i>p-trend</i>						0.04

¹ Conditional logistic regression models adjusted for body mass index, cigarette smoking status, parity, duration of oral contraceptive use, and duration of menopausal hormone therapy use.

² Less than 20% of marker values were above lower limit of detection, results should be interpreted with caution.

Table 3

Associations of *a priori* selected pre-diagnostic circulating inflammation markers and ovarian cancer risk by serous and non-serous histology, nested case-control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial.

	Serous Cases		Controls		Non-Serous Cases		Controls		OR ¹	95% CI	N=66	N=76	OR ²	95% CI
	N=83	N=88	N=83	N=88	N=66	N=76	N=66	N=76						
CRP (mg/L) < 3.23	20	24.1	31	35.2	1.00	(ref)	19	28.8	26	34.2	1.00	(ref)		
3.23-9.76	26	31.3	31	35.2	1.68	(0.60-4.74)	21	31.8	23	30.3	1.76	(0.67-4.60)		
> 9.76	37	44.6	26	29.6	3.96	(1.41-11.14)	26	39.4	27	35.5	2.13	(0.75-6.05)		
<i>p-trend</i>					0.008						0.22			
IL-1α (ng/L) ³ ≤ LLOD (3.2)	61	73.5	75	85.2	1.00	(ref)	54	81.8	65	85.5	1.00	(ref)		
Detectable	22	26.5	13	14.8	2.70	(1.10-6.63)	12	18.2	11	14.5	2.12	(0.66-6.88)		
IL-1β (ng/L) ≤ LLOD (0.64)	58	69.9	64	72.7	1.00	(ref)	52	78.8	60	79.0	1.00	(ref)		
Detectable	25	30.1	24	27.3	1.28	(0.52-3.18)	14	21.2	16	21.1	1.34	(0.54-3.36)		
IL-2 (ng/L) ≤ LLOD (0.64)	59	71.1	68	77.3	1.00	(ref)	53	80.3	64	84.2	1.00	(ref)		
Detectable	24	28.9	20	22.7	1.99	(0.80-4.99)	13	19.7	12	15.8	1.73	(0.62-4.76)		
IL-6 (ng/L) ≤ LLOD (0.64)	54	65.1	64	72.7	1.00	(ref)	46	69.7	57	75.0	1.00	(ref)		
Detectable	29	34.9	24	27.3	1.72	(0.76-3.91)	20	30.3	19	25.0	1.67	(0.68-4.07)		
IL-8 (ng/L) < 1.87	22	26.5	33	37.5	1.00	(ref)	19	28.8	23	30.3	1.00	(ref)		
1.87-3.79	23	27.7	26	29.6	1.61	(0.57-4.52)	20	30.3	30	39.5	0.68	(0.26-1.78)		
> 3.79	38	45.8	29	33.0	3.05	(1.09-8.51)	27	40.9	23	30.3	1.45	(0.47-4.54)		
<i>p-trend</i>					0.03						0.33			
IL-10 (ng/L) ≤ LLOD (0.64)	56	67.5	65	73.9	1.00	(ref)	48	72.7	58	76.3	1.00	(ref)		
Detectable	27	32.5	23	26.1	2.12	(0.84-5.36)	18	27.3	18	23.7	1.57	(0.64-3.87)		
IFN-γ (ng/L) ≤ LLOD (3.2)	67	80.7	69	78.4	1.00	(ref)	49	74.2	64	84.2	1.00	(ref)		
Detectable	16	19.3	19	21.6	1.07	(0.46-2.48)	17	25.8	12	15.8	4.42	(1.21-16.11)		
G-CSF (ng/L) ≤ LLOD (16.0)	50	60.2	53	60.2	1.00	(ref)	32	48.5	44	57.9	1.00	(ref)		
Detectable	33	39.8	35	39.8	1.44	(0.67-3.10)	34	51.5	32	42.1	1.23	(0.57-2.63)		
GM-CSF (ng/L) ≤ LLOD (13.2)	52	62.7	54	61.4	1.00	(ref)	34	51.5	45	59.2	1.00	(ref)		
Detectable	31	37.4	34	38.6	1.33	(0.62-2.82)	32	48.5	31	40.8	1.44	(0.63-3.26)		
TNF-α (ng/L) < 4.05	24	28.9	28	31.8	1.00	(ref)	12	18.2	26	34.2	1.00	(ref)		
4.05-5.48	26	31.3	30	34.1	1.13	(0.49-2.59)	33	50.0	25	32.9	4.92	(1.52-15.90)		

	Serous Cases		Controls		Non-Serous Cases		Controls		OR ²	95% CI
	N=83		N=88		N=66		N=76			
> 5.48	33	39.8	30	34.1	21	31.8	25	32.9	4.36	(1.11-17.05)
<i>p-trend</i>									0.047	

- ¹ Conditional logistic regression models adjusted for body mass index, cigarette smoking status, parity, duration of oral contraceptive use, and duration of menopausal hormone therapy use.
- ² Some matched sets include more than 1 case-control pair; therefore, in conditional logistic regression analyses restricted to specific tumor subtypes cases could have more than one matched control.
- ³ Less than 20% of marker values were above lower limit of detection, results should be interpreted with caution.

Table 4

Mutually adjusted models of the association of CRP, IL-8 and TNF- α and ovarian cancer risk, nested case-control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial.

	All Cases (n=149)		Serous Cases (n=83)		Non-Serous cases (n=66)		Analyses of cases with specimens collected <5 years prior to diagnosis (n=93)		Analyses of cases specimens collected 5+ years prior to diagnosis (n=56)	
	OR ^I	(95% CI)	OR ^I	(95% CI)	OR ^I	(95% CI)	OR ^I	(95% CI)	OR ^I	(95% CI)
CRP (mg/L) < 3.23	1.00	(ref)	1.00	(ref)	1.00	(ref)	1.00	(ref)	1.00	(ref)
3.23-9.76	1.38	(0.71-2.65)	1.63	(0.56-4.75)	2.90	(0.86-9.84)	1.41	(0.63-3.17)	1.53	(0.41-5.74)
> 9.76	2.06	(1.03-4.14)	4.26	(1.44-12.59)	2.39	(0.69-8.30)	2.15	(0.89-5.19)	6.04	(1.04-34.99)
<i>p-trend</i>	0.046		0.007		0.32		0.10		0.041	
IL-8 (ng/L) < 1.87	1.00	(ref)	1.00	(ref)	1.00	(ref)	1.00	(ref)	1.00	(ref)
1.87-3.79	1.08	(0.54-2.16)	1.94	(0.64-5.83)	0.38	(0.12-1.16)	1.13	(0.46-2.78)	1.05	(0.28-4.00)
> 3.80	1.78	(0.88-3.60)	3.66	(1.22-11.03)	0.93	(0.24-3.52)	2.26	(0.87-5.85)	1.52	(0.40-5.83)
<i>p-trend</i>	0.07		0.022		0.76		0.07		0.47	
TNF- α (ng/L) < 4.05	1.00	(ref)	1.00	(ref)	1.00	(ref)	1.00	(ref)	1.00	(ref)
4.05-5.48	1.72	(0.89-3.34)	0.88	(0.34-2.25)	6.99	(1.82-26.88)	1.09	(0.48-2.49)	6.24	(1.43-27.32)
> 5.49	1.64	(0.75-3.59)	1.22	(0.37-4.06)	4.42	(0.95-20.58)	1.05	(0.37-2.97)	5.74	(1.10-30.07)
<i>p-trend</i>	0.24		0.75		0.10		0.92		0.049	

^I Conditional logistic regression models adjusted for CRP, IL-8, TNF- α , body mass index, cigarette smoking status, duration of oral contraceptive use, and duration of menopausal hormone therapy use.

Exhibit 111

Pelvic Inflammatory Disease and the Risk of Ovarian Cancer and Borderline Ovarian Tumors

A Pooled Analysis of 13 Case-Control Studies

Christina B. Rasmussen; Susanne K. Kjaer; Vanna Albieri; Elisa V. Bandera; Jennifer A. Doherty; Estrid Høgdall; Penelope M. Webb; Susan J. Jordan; Mary Anne Rossing; Kristine G. Wicklund; Marc T. Goodman; Francesmary Modugno; Kirsten B. Moysich; Roberta B. Ness; Robert P. Edwards; Joellen M. Schildkraut; Andrew Berchuck; Sara H. Olson; Lambertus A. Kiemeny; Leon F. A. G. Massuger; Steven A. Narod; Catherine M. Phelan; Hoda Anton-Culver; Argyrios Ziogas; Anna H. Wu; Celeste L. Pearce; Harvey A. Risch; Allan Jensen | Am J Epidemiol. 2017;185(1):8-20.

Abstract and Introduction

Abstract

Inflammation has been implicated in ovarian carcinogenesis. However, studies investigating the association between pelvic inflammatory disease (PID) and ovarian cancer risk are few and inconsistent. We investigated the association between PID and the risk of epithelial ovarian cancer according to tumor behavior and histotype. We pooled data from 13 case-control studies, conducted between 1989 and 2009, from the Ovarian Cancer Association Consortium (OCAC), including 9,162 women with ovarian cancers, 2,354 women with borderline tumors, and 14,736 control participants. Study-specific odds ratios were estimated and subsequently combined into a pooled odds ratio using a random-effects model. A history of PID was associated with an increased risk of borderline tumors (pOR = 1.32, 95% confidence interval (CI): 1.10, 1.58). Women with at least 2 episodes of PID had a 2-fold increased risk of borderline tumors (pOR = 2.14, 95% CI: 1.08, 4.24). No association was observed between PID and ovarian cancer risk overall (pOR = 0.99, 95% CI: 0.83, 1.19); however, a statistically nonsignificantly increased risk of low-grade serous tumors (pOR = 1.48, 95% CI: 0.92, 2.38) was noted. In conclusion, PID was associated with an increased risk of borderline ovarian tumors, particularly among women who had had multiple episodes of PID. Although our results indicated a histotype-specific association with PID, the association of PID with ovarian cancer risk is still somewhat uncertain and requires further investigation.

Introduction

Ovarian cancer is the fifth most common cancer among women in developed countries, and it is the most fatal gynecological malignancy.^[1] The etiology of ovarian cancer is still not fully clarified, although a number of risk factors have been identified. A reduced risk of ovarian cancer has been observed with increased parity,^[2] use of oral contraceptives,^[2] hysterectomy,^[3] and tubal ligation,^[3] whereas family history of ovarian or breast cancer,^[2] use of hormone replacement therapy,^[2] exposure to talc,^[4] and a history of endometriosis^[5] have been associated with increased risks.

The 2 dominant hypotheses to explain the development of ovarian cancer relate increased risk to a large number of lifetime ovulatory cycles (the incessant ovulation theory)^[6] or exposure to high levels of gonadotropins (the gonadotropin theory).^[7] However, inflammation has also been suggested as a potential biological mechanism that may underlie a number of epidemiologic associations not easily explained by either theory,^[8,9] including talc exposure, endometriosis, tubal ligation, and hysterectomy. Furthermore, a link between pelvic inflammatory disease (PID) and the risk of ovarian cancer has been suggested, and this potential association may also be explained by the inflammation theory. PID is defined as an upper genital-tract infection and includes diagnoses of endometritis, salpingitis, pelvic peritonitis, and tubo-ovarian abscess caused by microorganisms ascending from the lower genital tract.^[10] Approximately 800,000 women are treated for PID annually in the United States,^[11] and it is estimated that 6%–20% of all women in the Western world are diagnosed with PID during their lifetimes.^[12–14]

Epidemiologic studies investigating the association between PID and the risk of ovarian cancer and borderline ovarian tumors have been inconsistent, revealing increased risks in some studies^[15–19] but not in all.^[20–23] Moreover, most previous studies have had methodological problems, including limited statistical power due to small numbers of study subjects and/or a short follow-up period. Also, ovarian cancer is a heterogeneous disease consisting of different histotypes with different risk factor profiles.^[24] However, few investigators have studied the role of PID separately for borderline tumors^[15,18] or for the separate histotypes of ovarian cancer.^[18,20]

To examine the association of PID with the risk of ovarian cancer, an international collaborative study was performed, using data from 13 case-control studies participating in the

Ovarian Cancer Association Consortium (OCAC). To our knowledge, this was the largest study of PID and ovarian cancer risk to date, thereby enabling a more robust estimation of risks among subgroups according to tumor behavior and histotype than has previously been possible.

Methods

Participating Studies

OCAC was founded in 2005 as an international forum of investigators conducting ovarian cancer case-control studies. The main aims of the collaboration are to discover associations between genetic polymorphisms and ovarian cancer risk and to identify and confirm epidemiologic risk factors for ovarian cancer.^[25]

For the present study, we obtained individual-level data from 13 case-control studies: 12 studies in OCAC^[20,26–37] and a parallel study not originally included in OCAC (Southern Ontario Ovarian Cancer Study (SON)).^[38] Eight studies were conducted in the United States (Connecticut Ovary Study (CON), Diseases of the Ovary and Their Evaluation (DOV), Hawaii Ovarian Cancer Study (HAW), Hormones and Ovarian Cancer Prediction (HOP), North Carolina Ovarian Cancer Study (NCO), New Jersey Ovarian Cancer Study (NJO), University of California Irvine Ovarian Cancer Study (UCI), and Los Angeles County Case-Control Studies of Ovarian Cancer (USC)).^[26,27,31–36] 2 in Canada (Familial Ovarian Tumor Study (TOR) and SON).^[37,38] 2 in Europe (Danish Malignant Ovarian Tumor Study (MAL) and Nijmegen Polygene Study and Nijmegen Biomedical Study (NTH)).^[28–30] and 1 in Australia (Australian Ovarian Cancer Study and Australian Cancer Study (Ovarian Cancer) (AUS)).^[20]

Characteristics of the 13 included studies are presented in . Data were cleaned and checked for internal consistency, and clarifications were obtained from the initial investigators if needed. Women with nonepithelial ovarian tumors ($n = 186$) and with missing information on PID status ($n = 278$) were excluded, leaving 9,162 women with invasive ovarian cancer (hereafter denoted "ovarian cancer"). 2,354 women with borderline ovarian tumors, and 14,736 control participants for analysis. Eleven studies included both women with ovarian cancer and women with borderline ovarian tumors, whereas 2 studies included only women with ovarian cancer (NTH and NJO). Each study had approval from the relevant institutional review board or ethics committee, and all participants gave informed consent.

Table 1. Characteristics of 13 Ovarian Cancer Case-Control Studies From the Ovarian Cancer Association Consortium, Conducted in Australia, Europe, and North America Between 1989 and 2009

First Author, Year (Reference No.)	Study Name and Acronym	Study Period	Study Type	Method of Data Collection	Age Range, years	Matching Variable	Mean Interval From Ovarian Cancer to Interview, months	Response Rate, %		Wording of Question Concerning PID Status	No. and % of Controls Who Had PID		Missing PID Data
								Cases	Controls		No.	%	
Australia													
Merritt, 2008 (20)	Australian Ovarian Cancer Study/Australian Cancer Study (Ovarian Cancer) (AUS)	2002–2005	Population-based	Self-administered questionnaire	18–80	Age (5-year categories)	5.3	84	47	Have you ever had pelvic inflammatory disease (e.g., chlamydia)? Have you ever had infection of the tubes or womb?	84	5.6	3.5
Europe													

Glud, 2004 (28)	Danish Malignant Ovarian Tumor Study (MAL)	1995–1999	Population-based	In-person interview	31–81	Age (5-year categories)	3.6	81	68	Have you ever been told by a doctor that you had pelvic inflammatory disease, that is an infection in your uterus or tubes? ^a	416	26.6	0.7
van Altena, 2012 (29)Wetzels, 2007 (30)	Nijmegen Polygene Study and Nijmegen Biomedical Study (NTH)	1989–2008	Population-based	Self-administered questionnaire	23–83	No matching	85.3	63	42	Could you tell whether you have ever had inflammation of the tubes or ovaries?	13	2.2	0.0
<i>North America</i>													
Risch, 2006 (34)	Connecticut Ovarian Cancer Study (CON)	1998–2003	Population-based	In-person interview	34–81	Age (3 age groups: 35–49 years, 50–64 years, and 65–79 years)	9.6	69	61	Could you tell me whether you have ever had an internal pelvic infection, sometimes called PID or pelvic inflammatory disease? We are not including bladder or vaginal infections in this.	23	4.2	0.2
Bodelon, 2012 (27)	Diseases of the Ovary and Their Evaluation (DOV)	2002–2009	Population-based	In-person interview	35–74	Age (5-year categories)	9.3	74	62	Before reference date, did a doctor or other health professional ever tell you that you had	65	3.5	0.3

Merritt, 2008 (20)	Australian Ovarian Cancer Study/Australian Cancer Study (Ovarian Cancer) (AUS)	2002–2005	Population-based	Self-administered questionnaire	18–80	Age (5-year categories)	5.3	84	47	Have you ever had pelvic inflammatory disease (e.g., chlamydia)? Have you ever had infection of the tubes or womb?	84	5.6	3.5
<i>Europe</i>													
Glud, 2004 (28)	Danish Malignant Ovarian Tumor Study (MAL)	1995–1999	Population-based	In-person interview	31–81	Age (5-year categories)	3.6	81	68	Have you ever been told by a doctor that you had pelvic inflammatory disease, that is an infection in your uterus or tubes? ^a	416	26.6	0.7
van Altena, 2012 (29)Wetzels, 2007 (30)	Nijmegen Polygene Study and Nijmegen Biomedical Study (NTH)	1989–2008	Population-based	Self-administered questionnaire	23–83	No matching	85.3	63	42	Could you tell whether you have ever had inflammation of the tubes or ovaries?	13	2.2	0.0
<i>North America</i>													
Risch, 2006 (34)	Connecticut Ovarian Cancer Study (CON)	1998–2003	Population-based	In-person interview	34–81	Age (3 age groups: 35–49 years, 50–64 years, and 65–79 years)	9.6	69	61	Could you tell me whether you have ever had an internal pelvic infection, sometimes called PID or pelvic inflammatory disease? We are not including bladder or vaginal	23	4.2	0.2

ordinal groups (age at first PID episode: <20, 20–29, or ≥30 years; time since first PID episode: <10, 10–19, or ≥20 years; number of PID episodes: 1 or ≥2), with women who had never had PID as the referent. Associations between the continuous variables (age at first PID episode and time since first PID episode) and ovarian cancer risk were assessed only among women who had ever been diagnosed with PID. In order to model these associations, we included PID status in the model as a categorical indicator variable together with the continuous PID variable, as suggested by Leffondré et al.^[41]

All analyses adjusted for age, parity (nulliparous vs. parous as well as parity as a continuous variable), oral contraceptive use (ever/never use as well as duration of use as a continuous variable), and family history of ovarian or breast cancer in a first-degree relative (yes/no) irrespective of their effect on the association between PID and ovarian cancer risk, because these factors were considered to be potentially important confounders a priori. For studies that used matching (age, race/ethnicity), conditional logistic regression analysis was used to adjust for these variables. In unmatched studies, age was categorized into 5-year age groups and unconditional logistic regression analysis was used (). When modeling parity and oral contraceptive use, the categorical variable was included as an indicator variable together with the continuous variable.^[41] Other potential confounders were considered but were not included in the final model, because none of them fulfilled an inclusion criterion of changing the log of the pooled estimate for ovarian cancer risk by 10% or more; these potential confounders were tubal ligation, hysterectomy, endometriosis, use of hormone replacement therapy, breastfeeding, age at menarche, menopausal status, body mass index, cigarette smoking, and educational level.

Table 1. Characteristics of 13 Ovarian Cancer Case-Control Studies From the Ovarian Cancer Association Consortium, Conducted in Australia, Europe, and North America Between 1989 and 2009

First Author, Year (Reference No.)	Study Name and Acronym	Study Period	Study Type	Method of Data Collection	Age Range, years	Matching Variable	Mean Interval From Ovarian Cancer to Interview, months	Response Rate, %		Wording of Question Concerning PID Status	No. and % of Controls Who Had Had PID		Missing PID Data
								Cases	Controls		No.	%	
Australia													
Merritt, 2008 (20)	Australian Ovarian Cancer Study/Australian Cancer Study (Ovarian Cancer) (AUS)	2002–2005	Population-based	Self-administered questionnaire	18–80	Age (5-year categories)	5.3	84	47	Have you ever had pelvic inflammatory disease (e.g., chlamydia)? Have you ever had infection of the tubes or womb?	84	5.6	3.5
Europe													
Glud, 2004 (28)	Danish Malignant Ovarian Tumor Study (MAL)	1995–1999	Population-based	In-person interview	31–81	Age (5-year categories)	3.6	81	68	Have you ever been told by a doctor that you had pelvic inflammatory disease, that is an infection	416	26.6	0.7

[illegible]

[illegible]

Ovarian Cancer (USC)	disease? That is, have you ever had an infection in your tubes? Before [month/year], did a doctor ever tell you that you had PID or pelvic inflammatory disease? ^b
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Abbreviation: PID, pelvic inflammatory disease.

^aStudies classified as having a requirement that the diagnosis of PID be verified by a physician.

bpopulation-based cases and non-population-based controls.

We examined interactions between PID status and parity (nulliparous vs. parous), oral contraceptive use (ever use vs. never use), and family history of ovarian or breast cancer in first-degree relatives (yes vs. no). Family history of breast or ovarian cancer was used as a proxy for hereditary ovarian cancer, as we aimed at exploring whether PID was similarly associated with hereditary and sporadic ovarian cancer. Linearity for all quantitative variables was examined by comparison with models with restricted cubic splines, but no appreciable deviations from linearity were found. The significances of the interactions and nonlinear associations were estimated by likelihood ratio tests of the interactions/nonlinearities and then comparison of the distribution of the study-specific P values with a uniform distribution by means of the Kolmogorov-Smirnov test.^[42]

All analyses were performed separately for ovarian cancer and for borderline tumors, and subgroup analyses were conducted by histotype. Ovarian cancers were divided into categories of serous, mucinous, endometrioid, clear cell, and other (including mixed cell, undifferentiated, and tumors of unknown epithelial histology). Additionally, serous cancers were divided into low-grade (grade 1) and high-grade (grade 2 or higher) tumors, because these are considered to represent different histotypes.^[43] However, 2 studies had no information on grade (SON and TOR) and were therefore not included in these analyses; they were included only in the analyses for serous cancer overall. Subgroup analyses for borderline ovarian tumors included serous and mucinous tumors, because other histotypes of borderline ovarian tumors are rare. All *P* values were 2-sided, and the nominal level of statistical significance was set at $P < 0.05$. All statistical analyses were performed using the statistical software R, version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria), including the packages "survival," "meta," and "rms."

Results

A history of PID was reported by 500 of the 9,162 women with ovarian cancers (5.5%), by 201 of the 2,354 women with borderline ovarian tumors (8.5%), and by 944 of the 14,736 control participants (6.4%). The proportion of control participants with PID varied across study sites, from 0.4% to 26.6%. In 11 of the studies, small proportions (less than 6%) of control participants reported PID, whereas in a Canadian study (SON) and in the Danish study (MAL), larger proportions of the control participants reported having had PID (20.2% and 26.6%, respectively). Median age at first PID episode was 28 years (interquartile range, 22–36 years) among women with ovarian cancer, 24 years (interquartile range, 20–30 years) among women with borderline ovarian tumors, and 25 years (interquartile range, 20–33 years) among control participants. Distributions of the various histotypes of ovarian tumors from the included studies are provided in (available at <http://aje.oxfordjournals.org/>).

Web Table 1. Number of Cases and Controls and Distribution of Histotypes by Study Site from the Ovarian Cancer Association Consortium (OCAC)

Continent	Study acronym	Controls	Ovarian cancer	Borderline ovarian tumors
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		All	Serous (%) ^a		Mucinous (%) ^a		Endometrioid (%) ^a		Clear cell (%) ^a		Other (%) ^a		All	Serous (%) ^b		Mucinous (%) ^b	
Australia	AUS	1,486	681	(61.4)	43	(3.9)	128	(11.5)	85	(7.7)	172	(15.5)	307	145	(47.2)	148	(48.2)
	MAL	1,557	339	(61.9)	50	(9.1)	74	(13.5)	43	(7.8)	42	(7.7)	200	104	(52.0)	86	(43.0)
	NTH	600	263	(45.2)	34	(12.9)	67	(25.5)	21	(8.0)	22	(8.4)					
North America	CON	551	373	(59.0)	19	(5.1)	74	(19.8)	35	(9.4)	25	(6.7)	108	69	(63.9)	36	(33.3)
	DOV	1,845	1,155	(58.2)	33	(2.9)	187	(16.2)	88	(7.6)	175	(15.2)	416	234	(56.3)	158	(38.0)
	HAW	1,103	709	(45.0)	71	(10.0)	117	(16.5)	82	(11.6)	120	(16.9)	186	88	(47.3)	91	(48.9)
	HOP	1,802	666	(54.7)	34	(5.1)	95	(14.3)	52	(7.8)	121	(18.2)	97	58	(58.0)	29	(29.9)
	NCO	1,083	862	(54.5)	43	(5.0)	138	(16.0)	87	(10.1)	124	(14.4)	224	155	(69.1)	64	(28.6)
	NJO	452	233	(56.7)	12	(5.2)	34	(14.6)	32	(13.7)	23	(9.9)					
	SON	557	362	(58.0)	39	(10.8)	70	(19.3)	29	(8.0)	14	(3.9)	83	42	(50.6)	39	(47.0)
	TOR	550	643	(65.5)	58	(9.0)	121	(18.8)	32	(5.0)	11	(1.7)	106	34	(32.1)	68	(64.2)
	UCI	561	383	(55.6)	26	(6.8)	67	(17.5)	35	(9.1)	42	(11.0)	194	120	(61.9)	73	(37.6)
	USC	2,589	1,856	(62.6)	162	(8.7)	238	(12.8)	119	(6.4)	175	(9.4)	433	249	(57.5)	178	(41.1)
Total		14,736	9,162	(58.1)	624	(6.8)	1,410	(15.4)	740	(8.1)	1,066	(11.6)	2,354	1,298	(55.1)	970	(41.2)

^aProportion of all ovarian cancers
^bProportion of all borderline ovarian tumors

Ovarian Cancer

In the pooled analysis, we found no association between a history of PID and the risk of ovarian cancer (odds ratio (OR) = 0.99, 95% confidence interval (CI): 0.83, 1.19) (and Figure 1). Furthermore, we observed no convincing associations of the age at first PID episode, time since first PID episode, or number of PID episodes with the risk of ovarian cancer ().

Web Table 2. Adjusted Pooled Odds Ratios and 95% Confidence Intervals for the Association Between Pelvic Inflammatory Disease and Ovarian Cancer in the Ovarian Cancer Association Consortium (OCAC)

	Studies (n)	Controls (n)	Overall			Serous			Serous low-grade			Serous high-grade			Mucinous			Endometriosis (n)	
			Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI		
PID status	13																		
Never PID		13,792	8662	1.00	Referent	5,036	1.00	Referent	320	1.00	Referent	3,657	1.00	Referent	592	1.00	Referent	1,328	1.00
Ever PID		944	500	0.99	0.83, 1.19	286	0.95	0.82, 1.11	44	1.48	0.92, 2.38	168	0.89	0.74, 1.08	32	0.84	0.56, 1.25	82	1.15

[illegible]

≥2		71	52	1.11	0.47, 2.65	23	1.06	0.49, 2.32	NA ^e	2	0.39	0.09, 1.7	5	1.29	0.37, 4.56	15	2.00
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Abbreviations: CI, confidence interval; NA, not applicable; PID, pelvic inflammatory disease; pOR, pooled odds ratio

^aNumbers may not add up due to missing values

^bAdjusted for parity (ever/never and number of pregnancies), oral contraceptive use (ever/never and duration of use) and family history of ovarian or breast cancer (yes/no)

^cAmong women with a history of PID

^dIn the statistical analysis for this particular category, statistically significant heterogeneity across the included studies were observed as *P* for heterogeneity was < 0.05

^eNot applicable due to insufficient numbers

Web Table 2. Adjusted Pooled Odds Ratios and 95% Confidence Intervals for the Association Between Pelvic Inflammatory Disease and Ovarian Cancer in the Ovarian Cancer Association Consortium (OCAC)

	Studies (n)	Controls (n)	Overall			Serous			Serous low-grade			Serous high-grade			Mucinous			Endometriosis	
			Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b
PID status	13																		
Never PID		13,792	8662	1.00	Referent	5,036	1.00	Referent	320	1.00	Referent	3,657	1.00	Referent	592	1.00	Referent	1,328	1.00
Ever PID		944	500	0.99	0.83, 1.19	286	0.95	0.82, 1.11	44	1.48	0.92, 2.38	168	0.89	0.74, 1.08	32	0.84	0.56, 1.25	82	1.15
Age at first PID (years)	12																		
Never PID		12,716	7976	1.00	Referent	4,724	1.00	Referent	309	1.00	Referent	3,384	1.00	Referent	524	1.00	Referent	1,217	1.00
<20		173	63	0.85 ^d	0.44, 1.64	37	1.05	0.65, 1.70	12	3.17	0.75, 13.38	21	0.92	0.55, 1.55	6	1.19	0.46, 3.12	11	1.49
20–29		359	161	0.84	0.68, 1.03	95	0.84	0.66, 1.08	15	0.98	0.54, 1.77	55	0.77	0.57, 1.05	15	1.32	0.74, 2.34	23	0.91
≥30		293	185	1.11	0.90, 1.36	102	1.02	0.79, 1.31	11	2.24 ^d	0.50, 10.00	53	1.06	0.77, 1.46	6	0.97	0.40, 2.34	34	1.53
<i>P trend</i>				0.08			0.13			0.82			0.10			0.87			0.27
per 1 year ^c				1.01	1.00, 1.03		1.01	1.00, 1.03		0.99	0.93, 1.06		1.02	1.00, 1.04		1.00	0.95, 1.05		1.02
Time since first PID	12																		

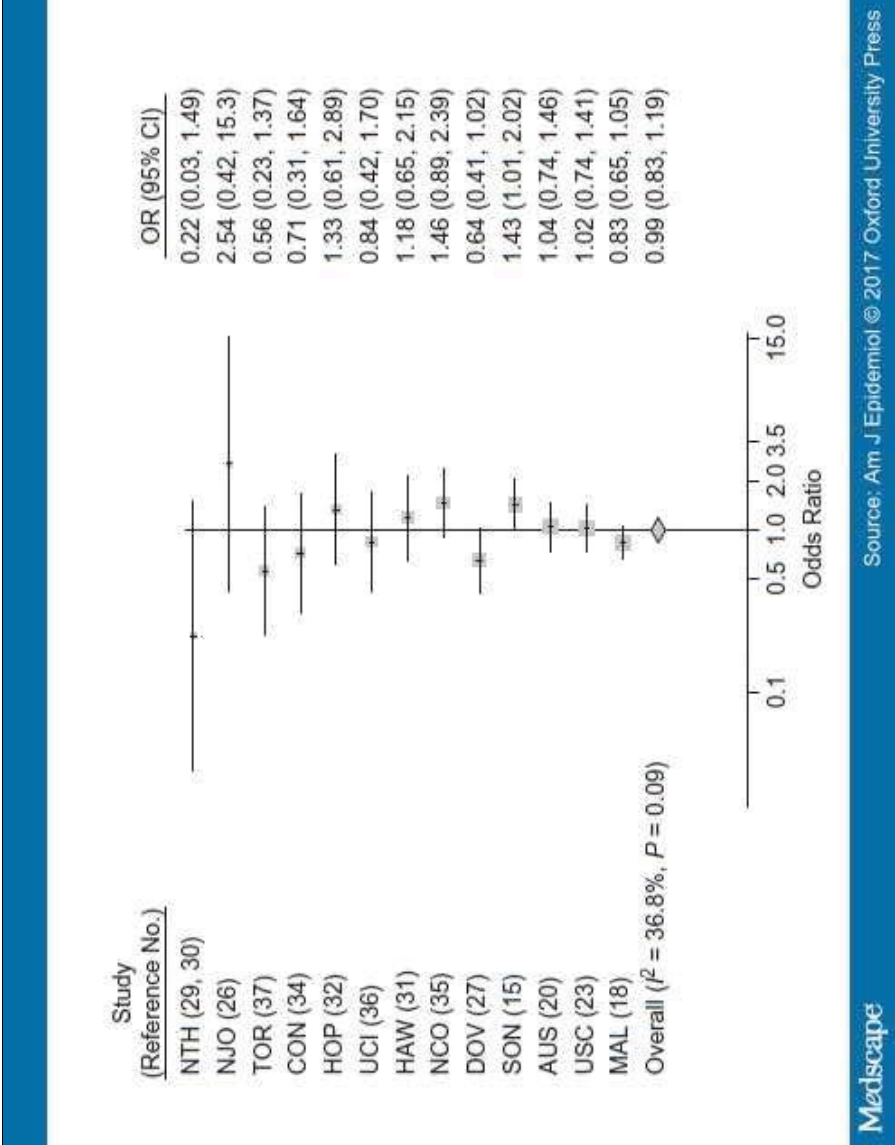


Figure 1.

Associations between pelvic inflammatory disease (PID) status and the risk of ovarian cancer among the participants of 13 case-control studies in Australia, Europe, and North America, conducted between 1989 and 2009. Results are presented according to study site and overall and are adjusted for age, parity, oral contraceptive use (ever/never use and duration of use), and family history of ovarian or breast cancer (yes/no). For 4 of the studies (AUS, MAL, SON, and USC), results for the association between PID and ovarian cancer risk have been published previously (15,18,20,23). For the remaining 9 studies, results for the association between PID and ovarian cancer risk have not been published previously, and their references therefore refer to papers with general information about these studies (26,27,29–32,34–37). For the present study, we obtained individual-level data from all 13 studies directly from the Ovarian Cancer Association Consortium database. Each square and line represent the odds ratio (OR) and 95% confidence interval (CI), respectively, and the size of the square indicates the study weighting. AUS, Australian Ovarian Cancer Study and Australian Cancer Study (Ovarian Cancer); CON, Connecticut Ovarian Cancer Study; DOV, Diseases of the Ovary and Their Evaluation; HAW, Hawaii Ovarian Cancer Study; HOP, Hormones and Ovarian Cancer Prediction; MAL, Danish Malignant Ovarian Tumor Study; NCO, North Carolina Ovarian Cancer Study; NJO, New Jersey Ovarian Cancer Study; NTH, Nijmegen Polygene Study and Nijmegen Biomedical Study; SON, Southern Ontario Ovarian Cancer Study; TOR, Familial Ovarian Tumor Study; UCI, University of California Irvine Ovarian Cancer Study; USC, Los Angeles County Case-Control Studies of Ovarian Cancer.

The magnitudes of the risk estimates for associations of specific histotypes of ovarian cancer with the individual PID variables did not differ from those observed for ovarian cancer

overall, and only a few of the risk estimates reached statistical significance. However, we noted a higher risk of low-grade serous cancer (OR = 1.48, 95% CI: 0.92, 2.38) associated with PID status, although the risk estimate did not reach statistical significance ().

Web Table 2. Adjusted Pooled Odds Ratios and 95% Confidence Intervals for the Association Between Pelvic Inflammatory Disease and Ovarian Cancer in the Ovarian Cancer Association Consortium (OCAC)

	Studies (n)	Controls (n)	Overall			Serous			Serous low-grade			Serous high-grade			Mucinous			Endometriosis	
			Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b
PID status	13																		
Never PID		13,792	8662	1.00	Referent	5,036	1.00	Referent	320	1.00	Referent	3,657	1.00	Referent	592	1.00	Referent	1,328	1.00
Ever PID		944	500	0.99	0.83, 1.19	286	0.95	0.82, 1.11	44	1.48	0.92, 2.38	168	0.89	0.74, 1.08	32	0.84	0.56, 1.25	82	1.15
Age at first PID (years)	12																		
Never PID		12,716	7976	1.00	Referent	4,724	1.00	Referent	309	1.00	Referent	3,384	1.00	Referent	524	1.00	Referent	1,217	1.00
<20		173	63	0.85 ^d	0.44, 1.64	37	1.05	0.65, 1.70	12	3.17	0.75, 13.38	21	0.92	0.55, 1.55	6	1.19	0.46, 3.12	11	1.49
20–29		359	161	0.84	0.68, 1.03	95	0.84	0.66, 1.08	15	0.98	0.54, 1.77	55	0.77	0.57, 1.05	15	1.32	0.74, 2.34	23	0.91
≥30		293	185	1.11	0.90, 1.36	102	1.02	0.79, 1.31	11	2.24 ^d	0.50, 10.00	53	1.06	0.77, 1.46	6	0.97	0.40, 2.34	34	1.53
<i>P trend</i>				0.08			0.13			0.82			0.10			0.87			0.27
per 1 year ^c				1.01	1.00, 1.03		1.01	1.00, 1.03		0.99	0.93, 1.06		1.02	1.00, 1.04		1.00	0.95, 1.05		1.02
Time since first PID (years)	12																		
Never PID		12,716	7976	1.00	Referent	4,724	1.00	Referent	309	1.00	Referent	3,384	1.00	Referent	524	1.00	Referent	1,217	1.00
<10		86	57	1.30	0.89, 1.91	31	1.23	0.76, 1.97	3	2.81	0.58, 13.63	11	1.38	0.64, 2.98	3	1.18	0.32, 4.42	11	2.11

10–19		160	84	1.09	0.81, 1.48	42	1.01	0.69, 1.48	9	1.51	0.67, 3.43	19	1.00	0.58, 1.72	10	1.72	0.83, 3.56	18	1.75
≥20		579	268	0.92 ^d	0.72, 1.18	161	0.87	0.72, 1.06	26	1.46	0.80, 2.67	99	0.80	0.67, 0.95	14	0.78	0.43, 1.42	39	0.95
<i>P trend</i>				0.24			0.52			0.47			0.52			0.88			0.16
per 5-year ^c				0.97	0.92, 1.02		0.98	0.91, 1.05		1.06	0.91, 1.22		0.97	0.89, 1.06		1.02	0.83, 1.25		0.92
Number of PID episodes	5																		
Never PID		3,737	2626	1.00	Referent	1,575	1.00	Referent		1.00	Referent	828	1.00	Referent	151	1.00	Referent	458	1.00
1		143	85	0.85	0.56, 1.29	54	0.98	0.63, 1.53		NA ^e		19	1.16	0.54, 2.52	5	0.75	0.25, 2.23	13	1.08
≥2		71	52	1.11	0.47, 2.65	23	1.06	0.49, 2.32		NA ^e		2	0.39	0.09, 1.7	5	1.29	0.37, 4.56	15	2.00

Abbreviations: CI, confidence interval; NA, not applicable; PID, pelvic inflammatory disease; pOR, pooled odds ratio

^aNumbers may not add up due to missing values

^bAdjusted for parity (ever/never and number of pregnancies), oral contraceptive use (ever/never and duration of use) and family history of ovarian or breast cancer (yes/no)

^cAmong women with a history of PID

^dIn the statistical analysis for this particular category, statistically significant heterogeneity across the included studies were observed as *P* for heterogeneity was < 0.05

^eNot applicable due to insufficient numbers

Borderline Ovarian Tumors

A history of PID was associated with a higher risk of borderline ovarian tumors (OR = 1.32, 95% CI: 1.10, 1.58) (and Figure 2). Furthermore, women with 2 or more episodes of PID had a more than 2-fold higher risk of borderline ovarian tumors compared with women without a history of PID (OR = 2.14, 95% CI: 1.08, 4.24). We found no consistent trend in the risk of borderline tumors with age at first episode of PID (*P*-trend = 0.29) or time since first episode of PID (*P*-trend = 0.44).

Table 2. Adjusted Pooled Odds Ratios for the Association Between Pelvic Inflammatory Disease and Borderline Ovarian Tumors Among Participants in the Ovarian Cancer Association Consortium (Australia, Europe, and North America), 1989–2009

PID History	No. of Studies	No. of Controls	Overall			Serous Borderline Tumors			Mucinous Borderline Tumors		
			No. of Cases ^a	pOR ^b	95% CI	No. of Cases ^a	pOR ^b	95% CI	No. of Cases ^a	pOR ^b	95% CI
PID status	11										
Never had PID		12,755	2,153	1.00	Referent	1,184	1.00	Referent	891	1.00	Referent
Ever had PID		929	201	1.32	1.10, 1.58	114	1.43	1.14, 1.79	79	1.28	0.97, 1.68
Age at first PID episode, years	10										

Never had PID		11,679		1,976	1.00	Referent	1,101	1.00	Referent	804	1.00	Referent
<20		172		33	1.38	0.91, 2.09	16	1.28	0.73, 2.25	16	1.89	1.06, 3.35
20–29		355		87	1.52	1.17, 1.97	52	1.72	1.25, 2.38	32	1.60	0.94, 2.70
≥30		283		50	1.24	0.90, 1.73	27	1.38	0.89, 2.12	20	1.46	0.89, 2.40
<i>P</i> -trend					0.29			0.25			0.96	
Per 1-year increment ^c					0.99	0.97, 1.01		0.98	0.96, 1.01		1.00	0.97, 1.03
Time since first PID episode, years	10											
Never had PID		11,679		1,976	1.00	Referent	1,101	1.00	Referent	804	1.00	Referent
<10		86		18	1.44	0.76, 2.73	12	1.74	0.86, 3.53	5	3.05	1.11, 8.40
10–19		159		48	1.73	1.21, 2.49	21	1.62	0.98, 2.70	25	2.37	1.46, 3.87
≥20		565		104	1.29	1.01, 1.64	62	1.48	1.09, 2.02	38	1.27	0.86, 1.86
<i>P</i> -trend					0.44			0.60			0.92	
Per 5-year increment ^c					1.03	0.95, 1.12		1.03	0.89, 1.20		0.99	0.88, 1.12
No. of PID episodes	4											
0		3,287		662	1.00	Referent	349	1.00	Referent	282	1.00	Referent
1		142		25	0.88	0.55, 1.39	17	1.11	0.63, 1.95	8	0.84	0.33, 2.14
≥2		70		24	2.14	1.08, 4.24	12	3.28 ^d	0.86, 12.54	11	1.98	0.80, 4.88

Abbreviations: CI, confidence interval; PID, pelvic inflammatory disease; pOR, pooled odds ratio.

^aNumbers may not add up to totals due to missing values.^bAdjusted for parity (ever/never pregnant and number of pregnancies), oral contraceptive use (ever/never use and duration of use), and family history of ovarian or breast cancer (yes/no).^cAmong women with a history of PID.^d *P* for heterogeneity < 0.05.

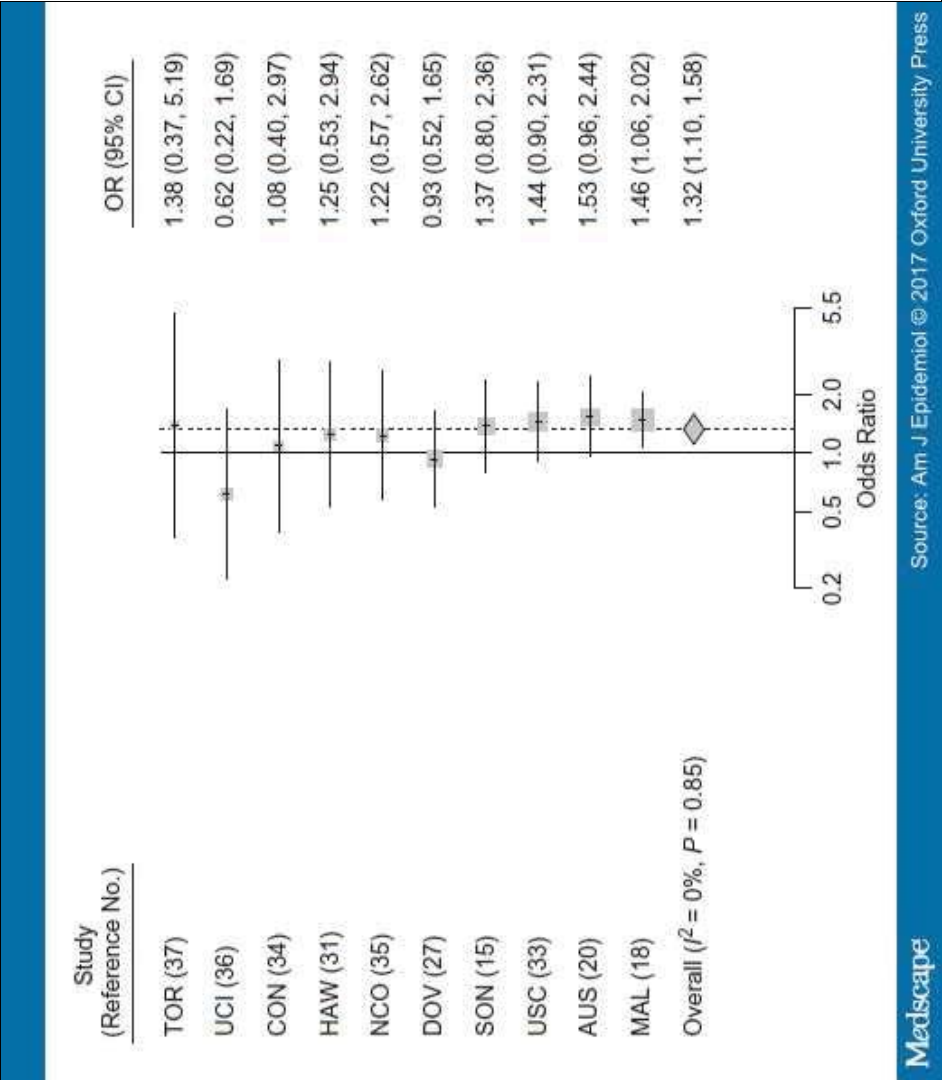


Figure 2.

Associations between pelvic inflammatory disease (PID) status and the risk of borderline ovarian tumors among the pooled participants of 13 case-control studies in Australia, Europe, and North America, conducted between 1989 and 2009. Results are presented according to study site and overall and are adjusted for age, parity, oral contraceptive use (ever/never use and duration of use), and family history of ovarian or breast cancer (yes/no). For 2 of the studies (MAL and SON) results for the association between PID and the risk of borderline ovarian tumors have been published previously (15, 18). For the remaining studies, results for the association between PID and the risk of borderline ovarian tumors have not been published previously, and their references therefore refer to papers with general information about these studies (20, 27, 31, 33–37). For the present study, we obtained individual-level data from all studies directly through the Ovarian Cancer Association Consortium database. Each square and line represent the odds ratio (OR) and 95% confidence interval (CI), respectively, and the size of the square indicates the study weighting. AUS, Australian Ovarian Cancer Study and Australian Cancer Study (Ovarian Cancer); CON, Connecticut Ovarian Cancer Study; DOV, Diseases of the Ovary and Their Evaluation; HAW, Hawaii Ovarian Cancer Study; HOP, Hormones and Ovarian Cancer Prediction; MAL, Danish Malignant Ovarian Tumor Study; NCO, North Carolina Ovarian Cancer Study; NJO, New Jersey Ovarian Cancer Study; NTH, Nijmegen Polygene Study and Nijmegen Biomedical Study; SON, Southern Ontario Ovarian Cancer Study; TOR, Familial Ovarian Tumor Study; UCI, University of California Irvine Ovarian Cancer Study; USC, Los Angeles County Case-Control Studies of Ovarian Cancer.

As for borderline ovarian tumors overall, the risk of serous borderline ovarian tumors was statistically significantly increased among women with PID (OR = 1.43, 95% CI: 1.14, 1.79). Similarly, PID was also associated with an increased risk of mucinous borderline ovarian tumors, although the risk estimate was not statistically significant (OR = 1.28, 95% CI: 0.97, 1.68). The risks of serous and mucinous borderline ovarian tumors were not convincingly associated with age at or time since first PID episode. In addition, women with multiple episodes of PID had a higher risk of both serous and mucinous borderline ovarian tumors, but none of the risk estimates reached statistical significance ().

Table 2. Adjusted Pooled Odds Ratios for the Association Between Pelvic Inflammatory Disease and Borderline Ovarian Tumors Among Participants in the Ovarian Cancer Association Consortium (Australia, Europe, and North America), 1989–2009

PID History	No. of Studies	No. of Controls	Overall			Serous Borderline Tumors			Mucinous Borderline Tumors		
			No. of Cases ^a	pOR ^b	95% CI	No. of Cases ^a	pOR ^b	95% CI	No. of Cases ^a	pOR ^b	95% CI
PID status	11										
Never had PID		12,755	2,153	1.00	Referent	1,184	1.00	Referent	891	1.00	Referent
Ever had PID		929	201	1.32	1.10, 1.58	114	1.43	1.14, 1.79	79	1.28	0.97, 1.68
Age at first PID episode, years	10										
Never had PID		11,679	1,976	1.00	Referent	1,101	1.00	Referent	804	1.00	Referent
<20		172	33	1.38	0.91, 2.09	16	1.28	0.73, 2.25	16	1.89	1.06, 3.35
20–29		355	87	1.52	1.17, 1.97	52	1.72	1.25, 2.38	32	1.60	0.94, 2.70
≥30		283	50	1.24	0.90, 1.73	27	1.38	0.89, 2.12	20	1.46	0.89, 2.40
<i>P</i> -trend				0.29			0.25			0.96	
Per 1-year increment ^c				0.99	0.97, 1.01		0.98	0.96, 1.01		1.00	0.97, 1.03
Time since first PID episode, years	10										
Never had PID		11,679	1,976	1.00	Referent	1,101	1.00	Referent	804	1.00	Referent
<10		86	18	1.44	0.76, 2.73	12	1.74	0.86, 3.53	5	3.05	1.11, 8.40
10–19		159	48	1.73	1.21, 2.49	21	1.62	0.98, 2.70	25	2.37	1.46, 3.87
≥20		565	104	1.29	1.01, 1.64	62	1.48	1.09, 2.02	38	1.27	0.86, 1.86
<i>P</i> -trend				0.44			0.60			0.92	
Per 5-year increment ^c				1.03	0.95, 1.12		1.03	0.89, 1.20		0.99	0.88, 1.12
No. of PID episodes	4										
0		3,287	662	1.00	Referent	349	1.00	Referent	282	1.00	Referent
1		142	25	0.88	0.55, 1.39	17	1.11	0.63, 1.95	8	0.84	0.33, 2.14
≥2		70	24	2.14	1.08, 4.24	12	3.28 ^d	0.86, 12.54	11	1.98	0.80, 4.88

Abbreviations: CI, confidence interval; PID, pelvic inflammatory disease; pOR, pooled odds ratio.

^aNumbers may not add up to totals due to missing values.

^bAdjusted for parity (ever/never pregnant and number of pregnancies), oral contraceptive use (ever/never use and duration of use), and family history of ovarian or breast cancer (yes/no).
^cAmong women with a history of PID.
^d *P* for heterogeneity < 0.05.

Additional Analyses

To consider the possibility that early cancer symptoms might have been misinterpreted as PID or that an episode of PID might have resulted in further examinations that led to the identification of ovarian cancer, we performed sensitivity analyses of the association between PID status and the risk of ovarian cancer and borderline ovarian tumors by excluding women whose last PID episode was ≤ 1 , ≤ 2 , or ≤ 3 years before the date of diagnosis of ovarian cancer (for cases) or date of interview (for controls). The risk estimates in these sensitivity analyses were not substantially different from the risk estimates in the main analyses (data not shown).

We performed additional sensitivity analyses by stratifying studies by data collection method (in-person interview vs. self-administered questionnaire), study continent (North America vs. Europe vs. Australia), whether a physician-verified diagnosis of PID was required, study period (before or including 2000 vs. after 2000), proportion of control participants with PID (low (<6%) vs. high (>20%)), body mass index (calculated as weight (kg)/height (m)²; <25 vs. ≥ 25), age at diagnosis of ovarian cancer (cases) or interview (controls) (<50 years vs. ≥ 50 years), and level of education (high school or less vs. more than high school). However, in the vast majority of these analyses, the direction and the magnitude of the associations were virtually unchanged compared with the associations obtained in the main analyses (data not shown). Notable exceptions were the observation of apparently statistically significantly increased risks of low-grade serous ovarian cancer (OR = 2.36, 95% CI: 1.24, 4.48) and endometrioid ovarian cancer (OR = 1.42, 95% CI: 1.01, 1.98) among women in the North American studies. However, no associations between PID and these 2 tumor types were found among the European studies or in the Australian study (low-grade serous cancer: pooled OR = 0.98, 95% CI: 0.61, 1.59 for the European studies and OR = 1.49, 95% CI: 0.52, 4.30 for the Australian study; endometrioid ovarian cancer: pooled OR = 0.60, 95% CI: 0.33, 1.10 for the European studies and OR = 1.09, 95% CI: 0.52, 2.26 for the Australian study).

Statistically significant heterogeneity across studies was observed for only a few of the risk estimates (and). However, additional analyses showed that neither the method of data collection nor study continent nor proportion of control participants with PID could explain the observed heterogeneity since these additional analyses did not reveal increased consistency among studies of the same type (data not shown). We observed no effect modification between PID status and any of the potential risk factors (parity, oral contraceptive use, and family history of ovarian/breast cancer) for ovarian cancer and borderline ovarian tumors (all *P* values > 0.05) (data not shown).

Web Table 2. Adjusted Pooled Odds Ratios and 95% Confidence Intervals for the Association Between Pelvic Inflammatory Disease and Ovarian Cancer in the Ovarian Cancer Association Consortium (OCAC)

	Studies (n)	Controls (n)	Overall			Serous			Serous low-grade			Serous high-grade			Mucinous			Endometri	
			Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b	95% CI	Cases ^a	pOR ^b
PID status	13																		
Never PID		13,792	8662	1.00	Referent	5,036	1.00	Referent	320	1.00	Referent	3,657	1.00	Referent	592	1.00	Referent	1,328	1.00
Ever PID		944	500	0.99	0.83, 1.19	286	0.95	0.82, 1.11	44	1.48	0.92, 2.38	168	0.89	0.74, 1.08	32	0.84	0.56, 1.25	82	1.15
Age at first PID (years)	12																		

Never PID		12,716	7976	1.00	Referent	4,724	1.00	Referent	309	1.00	Referent	3,384	1.00	Referent	524	1.00	Referent	1,217	1.00
<20		173	63	0.85 ^d	0.44, 1.64	37	1.05	0.65, 1.70	12	3.17	0.75, 13.38	21	0.92	0.55, 1.55	6	1.19	0.46, 3.12	11	1.49
20–29		359	161	0.84	0.68, 1.03	95	0.84	0.66, 1.08	15	0.98	0.54, 1.77	55	0.77	0.57, 1.05	15	1.32	0.74, 2.34	23	0.91
≥30		293	185	1.11	0.90, 1.36	102	1.02	0.79, 1.31	11	2.24 ^d	0.50, 10.00	53	1.06	0.77, 1.46	6	0.97	0.40, 2.34	34	1.53
<i>P trend</i>				0.08			0.13			0.82			0.10			0.87			0.27
per 1 year ^c				1.01	1.00, 1.03		1.01	1.00, 1.03		0.99	0.93, 1.06		1.02	1.00, 1.04		1.00	0.95, 1.05		1.02
Time since first PID (years)	12																		
Never PID		12,716	7976	1.00	Referent	4,724	1.00	Referent	309	1.00	Referent	3,384	1.00	Referent	524	1.00	Referent	1,217	1.00
<10		86	57	1.30	0.89, 1.91	31	1.23	0.76, 1.97	3	2.81	0.58, 13.63	11	1.38	0.64, 2.98	3	1.18	0.32, 4.42	11	2.11
10–19		160	84	1.09	0.81, 1.48	42	1.01	0.69, 1.48	9	1.51	0.67, 3.43	19	1.00	0.58, 1.72	10	1.72	0.83, 3.56	18	1.75
≥20		579	268	0.92 ^d	0.72, 1.18	161	0.87	0.72, 1.06	26	1.46	0.80, 2.67	99	0.80	0.67, 0.95	14	0.78	0.43, 1.42	39	0.95
<i>P trend</i>				0.24			0.52			0.47			0.52			0.88			0.16
per 5-year ^c				0.97	0.92, 1.02		0.98	0.91, 1.05		1.06	0.91, 1.22		0.97	0.89, 1.06		1.02	0.83, 1.25		0.92
Number of PID episodes	5																		
Never PID		3,737	2626	1.00	Referent	1,575	1.00	Referent		1.00	Referent	828	1.00	Referent	151	1.00	Referent	458	1.00
1		143	85	0.85	0.56, 1.29	54	0.98	0.63, 1.53		NA ^e		19	1.16	0.54, 2.52	5	0.75	0.25, 2.23	13	1.08
≥2		71	52	1.11	0.47, 2.65	23	1.06	0.49, 2.32		NA ^e		2	0.39	0.09, 1.7	5	1.29	0.37, 4.56	15	2.00

Abbreviations: CI, confidence interval; NA, not applicable; PID, pelvic inflammatory disease; pOR, pooled odds ratio

^aNumbers may not add up due to missing values
^bAdjusted for parity (ever/never and number of pregnancies), oral contraceptive use (ever/never and duration of use) and family history of ovarian or breast cancer (yes/no)
^cAmong women with a history of PID
^dIn the statistical analysis for this particular category, statistically significant heterogeneity across the included studies were observed as *P* for heterogeneity was < 0.05
^eNot applicable due to insufficient numbers

Table 2. Adjusted Pooled Odds Ratios for the Association Between Pelvic Inflammatory Disease and Borderline Ovarian Tumors Among Participants in the Ovarian Cancer Association Consortium (Australia, Europe, and North America), 1989–2009

PID History	No. of Studies	No. of Controls	Overall			Serous Borderline Tumors			Mucinous Borderline Tumors		
			No. of Cases ^a	pOR ^b	95% CI	No. of Cases ^a	pOR ^b	95% CI	No. of Cases ^a	pOR ^b	95% CI
PID status	11										
Never had PID		12,755	2,153	1.00	Referent	1,184	1.00	Referent	891	1.00	Referent
Ever had PID		929	201	1.32	1.10, 1.58	114	1.43	1.14, 1.79	79	1.28	0.97, 1.68
Age at first PID episode, years	10										
Never had PID		11,679	1,976	1.00	Referent	1,101	1.00	Referent	804	1.00	Referent
<20		172	33	1.38	0.91, 2.09	16	1.28	0.73, 2.25	16	1.89	1.06, 3.35
20–29		355	87	1.52	1.17, 1.97	52	1.72	1.25, 2.38	32	1.60	0.94, 2.70
≥30		283	50	1.24	0.90, 1.73	27	1.38	0.89, 2.12	20	1.46	0.89, 2.40
<i>P</i> -trend				0.29			0.25			0.96	
Per 1-year increment ^c				0.99	0.97, 1.01		0.98	0.96, 1.01		1.00	0.97, 1.03
Time since first PID episode, years	10										
Never had PID		11,679	1,976	1.00	Referent	1,101	1.00	Referent	804	1.00	Referent
<10		86	18	1.44	0.76, 2.73	12	1.74	0.86, 3.53	5	3.05	1.11, 8.40
10–19		159	48	1.73	1.21, 2.49	21	1.62	0.98, 2.70	25	2.37	1.46, 3.87
≥20		565	104	1.29	1.01, 1.64	62	1.48	1.09, 2.02	38	1.27	0.86, 1.86
<i>P</i> -trend				0.44			0.60			0.92	
Per 5-year increment ^c				1.03	0.95, 1.12		1.03	0.89, 1.20		0.99	0.88, 1.12
No. of PID episodes	4										
0		3,287	662	1.00	Referent	349	1.00	Referent	282	1.00	Referent
1		142	25	0.88	0.55, 1.39	17	1.11	0.63, 1.95	8	0.84	0.33, 2.14
≥2		70	24	2.14	1.08, 4.24	12	3.28 ^d	0.86, 12.54	11	1.98	0.80, 4.88

Abbreviations: CI, confidence interval; PID, pelvic inflammatory disease; pOR, pooled odds ratio.

^aNumbers may not add up to totals due to missing values.

^bAdjusted for parity (ever/never pregnant and number of pregnancies), oral contraceptive use (ever/never use and duration of use), and family history of ovarian or breast cancer (yes/no).

^cAmong women with a history of PID.

^d *P* for heterogeneity < 0.05.

Discussion

To our knowledge, this was the largest study to date to have investigated the association between history of PID and the risk of ovarian cancer. In a pooled analysis of 13 case-control studies, we found no convincing associations between self-reported PID status and the risk of ovarian cancer overall, but suggestions of an increased risk of low-grade serous cancer were noted. For borderline ovarian tumors, an increased risk was observed among women with a history of PID, both overall and for serous and mucinous borderline tumors separately. Furthermore, the risk of borderline tumors increased with the number of PID episodes.

An association between PID and the risk of ovarian tumors is biologically plausible and could be explained by the inflammation hypothesis.^[8] Inflammation is characterized by the production of free radicals, cytokines, prostaglandins, and growth factors with the potential for genetic and epigenetic changes to the DNA, resulting in an increased risk of malignant transformation.^[44] Until recently, it was believed that all histotypes of ovarian cancer arose from the mesodermal surface epithelium, either on peritoneal surfaces or entrapped within the ovaries, and inflammation of the epithelium was therefore proposed to trigger malignant transformation.^[8] Recently, it has been suggested that some serous ovarian tumors originate in the mucosal epithelium of the fallopian tube, and inflammation of the fallopian tubes has been proposed to contribute to the development of these tumors.^[45]

The association between PID and the risk of ovarian cancer has been investigated in only 2 cohort studies^[17,19] and 7 case-control studies.^[15,16,18,20–23] However, 4 of those case-control studies were based on data from study sites (MAL, USC, AUS, and SON) that were included in the present analysis;^(15,18,20,23) results from those studies will not be discussed further. We found a 32% higher risk of borderline ovarian tumors associated with a history of PID, and risk estimates above unity were noted for nearly all individual studies. Furthermore, we observed similarly increased risks of serous and mucinous borderline tumors associated with PID status. Our novel finding of a 2-fold higher risk among women with multiple PID episodes may reflect a true association between PID and the risk of borderline ovarian tumors rather than being caused by chance or bias. Only 2 studies (SON and MAL, both included in the present analyses) have previously investigated the association between PID and the risk of borderline tumors.^[15,18]

In the present study, the lack of any marked associations between PID and the risk of ovarian cancer overall is consistent with results from 1 case-control study,^[22] whereas 2 other studies found an increased risk of ovarian cancer.^[16,17] Additionally, 2 studies assessed PID in relation to ovarian cancer risk but provided results only for ovarian cancer and borderline tumors combined, thereby hampering a comparison with the present results.^[19,21] Ness et al.^[21] reported null findings, and McAlpine et al.^[19] in a Canadian cohort study, reported a 4-fold higher risk of ovarian cancer among women who had had PID. Concerning the histotypes of ovarian cancer, indications of an increased risk of low-grade serous cancer with PID were noted in the main analysis. Conversely, no convincing associations between PID and the risk of high-grade serous, mucinous, clear cell, or endometrioid ovarian cancer were noted in the main analyses. However, sensitivity analyses revealed statistically significantly increased risks of low-grade serous and endometrioid ovarian cancers when using data from the North American studies only. Other than 2 studies already included in the present pooled analysis, no previous studies have assessed the association between PID and the risk of ovarian cancer according to histotype. Although we cannot completely rule out the possibility that these histotype-specific findings may be due to chance, the present study is the first, to our knowledge, to indicate differences in the risk profile of ovarian cancer histotypes with regard to PID. However, the low number of exposed cases for most of the histotypes limited the precision of the risk estimates, and our results must therefore be confirmed by others.

Nevertheless, our results suggest that PID may be differentially associated with the risk of ovarian tumors. Reasons for this difference are not known, but they may be associated with different pathogeneses of the ovarian tumor histotypes. Recently, the so-called dualistic model of ovarian carcinogenesis proposed that borderline tumors are precursors of type 1 (low-grade) ovarian cancers but unrelated to type 2 (high-grade) ovarian cancers.^[46] According to this hypothesis, type 1 tumors include low-grade serous and mucinous carcinomas, and these are believed to develop along a continuum of tumor progression from adenoma to borderline tumor to invasive carcinoma.^[46] Clear cell and low-grade endometrioid carcinomas are also type 1 cancers and are believed to develop from endometriosis. Our results demonstrated an association between PID and the risk of borderline ovarian tumors and indicated that the risk of low-grade serous cancer might also be increased, which accords well with the theory of a stepwise development from a serous borderline tumor to low-grade serous cancer. In contrast, no associations between PID and high-grade serous ovarian cancer were observed. Therefore, our results suggest that PID is a risk factor for borderline and possibly also low-grade serous ovarian cancer, whereas no marked associations were observed for the other histotypes of ovarian cancer. The possible underlying

biological mechanisms responsible for this differential association between PID and ovarian tumor types are unknown and require further investigation in epidemiologic and biological studies.

A strength of the present study is the use of pooled data from 13 case-control studies. The large sample size resulted in increased statistical power and enabled us to estimate risks according to invasiveness and histotype. Moreover, all the studies we included were population-based, and information on PID was obtained through in-person interviews in the majority of them. In addition, we used individual-level data carefully harmonized and entered into a single data set. The use of a 2-stage approach^[39] enabled us to account for differences in design and data collection between studies and to control for several potential confounders. Finally, all studies with the relevant exposure data in OCAC were included regardless of their individual results, thus removing the influence of publication bias.

Some limitations should also be mentioned. First, information about PID status was self-reported in all studies, and the proportion of control participants reporting an episode of PID in the individual studies ranged from 0.4% to 27%. Unfortunately, most studies had no data or insufficient data on treatment for PID, which could have added important information in terms of validating the PID diagnoses. The highest frequencies were reported in the Danish study (MAL: 27%) and in a Canadian study (SON: 20%); the remaining 11 studies all had PID proportions below 6%. Reasons for the differences in proportions among the studies may include geographic variation in the prevalence of PID-causing pathogens, different phrasing of the PID-related questions, or differences in the prevalence of high-risk sexual behaviors. However, we believe that underestimation of PID exposure is the most likely cause for the low proportions of women with a history of PID in the majority of studies, because previous studies from Sweden and the United States have estimated lifetime prevalences of PID between 6% and 20%.^[12–14] In studies with self-reported data on PID exposure, including the present study, the true proportion of women who have had PID might be underestimated for several reasons—women might have forgotten about a past PID episode, chosen not to report it, or had unrecognized, subclinical PID. Hence, we cannot rule out the possibility that this misclassification of PID status could have influenced our results. Interestingly, investigators in only 2 previous studies did not use self-reported data on PID but instead obtained information on PID from a population-based health insurance database or used evidence of inflammation at surgery for tubal damage as a proxy for previous PID, and both groups reported an increased risk of ovarian cancer associated with PID.^[17, 9] Therefore, in future studies, researchers should consider using a more objective measure of PID, such as data obtained from reliable health registries or through serological testing for antibodies to PID-causing pathogens, including *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

Second, misclassification of PID exposure might also result when women mistakenly report bladder or vaginal infections as PID. However, we expect this misclassification to be relatively infrequent, because in the majority of included studies, PID was defined as diagnosed by a physician, or the question specified that bladder or vaginal infections were not included. Furthermore, the majority of studies performed in-person interviews, thus allowing for potential uncertainties to be clarified. Third, the retrospective design of case-control studies introduces the potential for recall bias, in which case patients are more likely than control participants to report past exposures. However, we would not expect such overreporting to be differential with respect to degree of invasiveness of diagnosed ovarian tumors, and we therefore do not believe that this can explain the increased risk we observed for borderline tumors but not for ovarian cancer. Fourth, surveillance bias is potentially of concern, because women with PID symptoms may undergo ultrasonography or laparoscopy during which the ovaries are visualized, leading to coincidental findings of ovarian tumors. However, this potential surveillance bias is probably minimal, because our sensitivity analyses excluding women with PID less than 1–3 years in the past revealed virtually identical results as in the main analyses. Fifth, only 5 studies had information on the number of PID episodes, and the absence of thorough information on this exposure variable limited our ability to fully investigate and interpret any potential dose-response associations between number of PID episodes and the risk of ovarian cancer and borderline ovarian tumors. Finally, despite the large study size, we still had limited statistical power because of small proportions of women with PID in some of the categorical analyses and for some of the rarer histotypes, and we cannot completely rule out the possibility that some of the observed associations may have been due to the large number of comparisons; thus our results should be interpreted with caution.

In conclusion, in this large, pooled analysis, we observed an increased risk of borderline ovarian tumors among women with a history of PID. These risks increased with the number of PID episodes. Conversely, we found no association between PID and the risk of ovarian cancer overall, but indications of an increased risk of low-grade serous cancer were noted. These findings suggest that PID may be a risk factor for borderline ovarian tumors and possibly for low-grade serous cancer, although no convincing associations were seen for other ovarian cancer histotypes. However, until the specificity of the association is confirmed in additional epidemiologic and biological studies, the association between PID and ovarian cancer risk is still somewhat uncertain.

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Abbreviations

AUS, Australian Ovarian Cancer Study/Australian Cancer Study (Ovarian Cancer); CI, confidence interval; CON, Connecticut Ovarian Cancer Study; DOV, Diseases of the Ovary and Their Evaluation; HAW, Hawaii Ovarian Cancer Study; HOP, Hormones and Ovarian Cancer Prediction; MAL, Danish Malignant Ovarian Tumor Study; NCO, North Carolina Ovarian Cancer Study; NJO, New Jersey Ovarian Cancer Study; NTH, Nijmegen Polygene Study and Nijmegen Biomedical Study; OCAC, Ovarian Cancer Association Consortium; OR, odds ratio; PID, pelvic inflammatory disease; pOR, pooled odds ratio; SON, Southern Ontario Ovarian Cancer Study; TOR, Familial Ovarian Tumor Study; UCI, University of California Irvine Ovarian Cancer Study; USC, Los Angeles County Case-Control Studies of Ovarian Cancer.

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3

Prevention and Early Detection

Improving the prevention and early detection of ovarian carcinomas will be a critical component of reducing morbidity and mortality from ovarian cancer. This chapter discusses the genetic and nongenetic risk factors of the disease along with potential prevention strategies and methods for early detection and screening of ovarian cancer. In particular, this chapter identifies a number of gaps in knowledge related to identifying those women who are at highest risk for developing ovarian carcinomas, and it describes several challenges to developing screening tests for high-risk women, their families, and the general population. The chapter also explains how gaps in knowledge about the basic biology of ovarian carcinomas (as discussed in Chapter 2) hinder the development of better methods to prevent ovarian carcinomas or detect them at the earliest stage of disease progression.

RISK ASSESSMENT FOR OVARIAN CANCER

Although scientists' understanding about the early carcinogenic events of ovarian cancer is incomplete (see Chapter 2), researchers have identified several factors associated with either an increased or a decreased risk of developing ovarian cancer (see Table 3-1). While some of these risks factors cannot be modified (e.g., age and ancestry), a number of others (e.g., hormone use and diet) can be altered through lifestyle changes, pharmacological interventions, or surgery. A critical drawback, however, is that nearly all of the identified risk factors are associated predominantly with the less common and less lethal ovarian cancer subtypes and not with the most common and lethal type—high-grade serous carcinoma (HGSC). Ovarian

Smoking

The association of smoking with risk for ovarian cancer varies by subtype. A recent meta-analysis found a 7 percent increased risk of ovarian cancer for current smokers versus women who had never smoked, but the association varied significantly by histologic subtype (Beral et al., 2012). Smoking was associated with an approximately 20 percent lower risk for endometrioid and clear cell carcinomas and an approximately 80 percent increased risk for mucinous carcinomas among current smokers versus never-smokers; serous carcinomas were not associated with smoking.

Inflammation

Studies of the inflammatory marker C-reactive protein suggest a possible association between inflammation and an increased risk of ovarian cancer (Ose et al., 2015b; Poole et al., 2013). Other specific inflammatory factors have also been associated with ovarian cancer. A meta-analysis reported that exposure to asbestos was associated with a 77 percent increased risk of ovarian cancer mortality (Camargo et al., 2011), and the International Agency for Research on Cancer determined that there was sufficient evidence to support a causal relationship between asbestos exposure and ovarian cancer (Straif et al., 2009). This has led to studies of talc use, which is chemically similar to asbestos and can cause an inflammatory response. The use of perineal talcum powder has been associated with a 20 to 30 percent increased risk of ovarian cancer, although it also has been shown to vary by histologic subtype (Cramer et al., 2015; Terry et al., 2013). One analysis reported a 9 percent lower ovarian cancer risk with regular aspirin use, with stronger results among daily users (Trabert et al., 2014). However, most cohort studies have not observed a similar reduction in risk (Brasky et al., 2014; Lacey et al., 2004; Murphy et al., 2012; Ni et al., 2013; Pinheiro et al., 2009; Prizment et al., 2010; Setiawan et al., 2012).

As mentioned previously, endometriosis is associated with an increased risk of ovarian cancer, and tubal ligation and hysterectomy (which may limit the ability of endometrial tissues to access the fallopian tubes, ovaries, and pelvic region by retrograde menstruation) act to decrease this risk. Hysterectomy is associated with an approximately 30 percent decreased risk of ovarian cancer, and tubal ligation has been associated with a 26 percent decreased risk of ovarian cancer overall and a 55 percent lower risk of endometrioid cancer, the ovarian cancer type most strongly associated with endometriosis (Rice et al., 2012). The exact causes for the increased risk of ovarian cancer from endometriosis are unknown. However, endometriosis is associated with an inflammatory environment characterized by elevated levels of cytokines and growth factors (Arici, 2002; Malutan

Exhibit 113



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Review Article

Updates of the role of oxidative stress in the pathogenesis of ovarian cancer

Ghassan M. Saed^{a,*}, Michael P. Diamond^b, Nicole M. Fletcher^a^a The Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI, United States^b The Department of Obstetrics and Gynecology, Augusta University, Augusta, GA, United States

HIGHLIGHTS

- Oxidative stress plays an essential role in the pathogenesis of ovarian cancer.
- Modulating the redox balance may have therapeutic value.
- Chemoresistant ovarian cancer cells have an even further elevated oxidative stress.
- Chemotherapy-induced mutations in redox enzymes may contribute to chemoresistance.

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ABSTRACT

Clinical and epidemiological investigations have provided evidence supporting the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS), collectively known as oxidative stress, in the etiology of cancer. Exogenous factors such as chronic inflammation, infection and hypoxia are major sources of cellular oxidative stress. Specifically, oxidative stress plays an important role in the pathogenesis, neoangiogenesis, and dissemination of local or distant ovarian cancer, as it is known to induce phenotypic modifications of tumor cells by cross talk between tumor cells and the surrounding stroma. Subsequently, the biological significance of the relationship between oxidative stress markers and various stages of epithelial ovarian cancer highlights potential therapeutic interventions as well as provides urgently needed early detection biomarkers. In the light of our scientific research and the most recent experimental and clinical observations, this review provides the reader with up to date most relevant findings on the role of oxidative stress in the pathogenesis of ovarian cancer and the possible therapeutic implications.

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* Corresponding author at: Wayne State University, 275 E Hancock Street, Detroit, MI 48201, United States.
E-mail address: gsaed@med.wayne.edu (G.M. Saed).

1. Ovarian cancer

Ovarian cancer is the fifth leading cause of cancer death; the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy; yet the underlying pathophysiology continues to be delineated [1]. The majority of advanced-stage tumors are of epithelial cell origin and can arise from serous, mucinous, or endometrioid cells on the surface epithelium of the ovary or the fallopian tube [1]. Surgical cytoreduction followed by platinum/taxane chemotherapy results in complete clinical response in 50–80% of patients with stage III and IV disease, but most will relapse within 18 months with chemoresistant disease [1]. Mortality rates for this type of malignancy are high because of a lack of an early-stage screening method, as well as the development of drug resistance [1].

Many cases of ovarian cancer continue to be described as *de novo* although several theories regarding its origination have been proposed. Some of these theories include 1) the incessant ovulation hypothesis, where ovarian surface epithelial cells are injured due to repeated ovulation leading to eventual transformation and malignancy, 2) the gonadotropin hypothesis describes overstimulation of ovarian surface epithelium through hormone receptors leading to malignant transformation, and 3) the cell of origin for most epithelial ovarian cancer is not originating in the ovary but rather coming from the fallopian tube and spreading to the ovary, and beyond [1–3]. Thus, the exact origin(s) and pathogenesis of ovarian cancer still remains under debate.

Recently, a revised model of epithelial ovarian carcinogenesis has been proposed that distinguishes more clearly between type I and type II tumors based on both molecular genetic findings and histopathologic studies [3]. Kurman and Shih describe a dualistic model of ovarian carcinogenesis where type I tumors develop from benign extraovarian precursor lesions that implant on the ovary are classified into three groups described as; endometriosis-related tumors (endometrioid, clear cell, and seromucinous), low-grade serous carcinomas, and then mucinous carcinomas and malignant Brenner tumors [3]. On the other hand, type II tumors develop from intraepithelial carcinomas in the fallopian tube, and involve both the ovary and extraovarian sites and are classified as high-grade serous carcinomas that can be further subdivided into morphologic and molecular subtypes [3].

The overwhelming majority of ovarian cancers are derived from ovarian surface epithelium. Metastasis is achieved through detachment of single cells or clusters of cells from the primary tumor followed by implantation on peritoneal mesothelial lining [4]. Unlike many other type of cancer, ovarian carcinomas rarely metastasize outside of the peritoneal cavity [5]. Additionally, the presence of spheroids in ascites is a contributing factor to not only metastasis but also to chemoresistance. Spheroid cells are also known as ovarian cancer stem cells that have numerous characteristics of cancer stem cells including self-renewal, the ability to produce differentiated progeny, increased expression of genes associated with cancer stem cells, higher invasiveness, migration potential, altered metabolism, and enhanced chemoresistance [4,6].

Ovarian cancer has also been characterized to manifest loss of function of the p53 gene due to mutations as well as other oncogenic pathways including retinoblastoma protein, the phosphatidylinositol 3 kinase (PI3K)/rat sarcoma viral oncogene pathways, and Notch signaling [1]. Moreover, ovarian cancer is associated with germline mutations in the *BRCA1* or *BRCA2* genes, affecting only 20–40% of patients, suggesting the possibility of the presence of unknown mutations in other genes [1]. Additional genetic variations, many of which have been identified in recent genome-wide association studies, have been hypothesized to act as low to moderate penetrant alleles, which contribute to ovarian cancer risk, as well as other diseases [7]. Several studies have been done to identify differentially expressed genes in ovarian carcinoma for diagnosis of early-stage ovarian cancer as well as the use of such markers as targets for improved therapy and treatment, although to date these

have not yielded reproducible prognostic indicators for identification and clinical outcomes [1,8–10].

2. Oxidative stress

The imbalance between production and elimination of free radicals and reactive metabolites leads to a state of oxidative stress and subsequent damage of important biomolecules and cells, with potential impact on the whole organism [11]. Reactive oxygen species (ROS) are oxygen-derived small molecules, including oxygen radicals, such as superoxide ($O_2^{\bullet-}$), hydroxyl (HO^{\bullet}), peroxy (RO_2^{\bullet}), and alkoxy (RO^{\bullet}), as well as various non-radicals that can be converted to radicals or serve as oxidizing agents and include hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), ozone (O_3), and singlet oxygen (1O_2) [11, 12]. Reactive nitrogen species (RNS) are nitrogen-containing oxidants and are formed from nitric oxide (NO) that is generated from the mitochondrial respiratory chain under hypoxic conditions [11]. The persistent generation of cellular ROS and RNS is a consequence of many factors including exposure to carcinogens, infection, inflammation, environmental toxicants, nutrients, and mitochondrial respiration [11–14]. Various enzyme systems produce ROS and RNS including cytochrome P450, lipoxygenase, cyclooxygenase, nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase complex, xanthine oxidase (XO), and peroxisomes [11,13,15] (Fig. 1).

Various enzyme systems that neutralize toxic ROS and RNS are vital in maintaining the redox balance, and are summarized in Fig. 1. Superoxide dismutase (SOD) catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2 , which then can be converted to water by catalase (CAT) or glutathione peroxidase (GPX) coupled with glutathione reductase (GSR) [12] (Fig. 1). Other important scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which utilizes glutathione (GSH) as a substrate. Additionally, glutathione S-transferase (GST) is involved in detoxification of varieties of environmental carcinogens and xenobiotics by catalyzing their conjugation to GSH, and subsequent removal from the cell [12] (Fig. 1). Glutathione plays a central role in maintaining redox homeostasis, and the GSH-to-oxidized-GSH (GSH/GSSG) ratio provides an estimate of cellular redox buffering capacity [16,17]. Moreover, evidence suggests that increased oxidative stress mediated by the GSH/GSSG complex results in enhanced activity of the GS-X-MRP1 efflux pump [17]. This pump is known to decrease the intracellular effective chemotherapeutic drug concentration; therefore it is considered one of the mechanisms of multiple drug resistance [16, 17].

3. Oxidative stress and cancer

Oxidative stress has been reported to affect all phases of the oncogenic process including initiation, promotion, and progression [11,12]. Oxidative stress is known to activate several transcription factors including nuclear factor (NF)- κ B, activator protein (AP)-1, p53, hypoxia inducible factor (HIF)-1 α , peroxisome proliferator-activated receptor (PPAR)- γ , β -catenin/Wnt, and Nuclear factor erythroid 2-related factor 2 (Nrf2), which modulate the expression of numerous genes involved in immune and inflammatory responses, tissue remodeling and fibrosis, carcinogenesis, and metastasis [11]. The expression of some antioxidant enzymes is known to be controlled by the master transcription factor regulator Nrf2 [11,18]. The activation of Nrf2 involves a suppressor protein known as Kelch Like ECH Associated Protein 1 (Keap1) that binds Nrf2 in the cytoplasm, preventing its translocation into the nucleus for binding specific promoters [11,18].

Reactive oxygen species are known to alter the expression of several genes through induction of genetic mutations, resulting in alteration of the balance between cell proliferation and apoptosis [1,11,19]. Damage to DNA by ROS is now accepted as a major cause of cancer, and has been demonstrated in both breast and hepatocellular carcinoma [20]. Oxidation of DNA bases, such as thymidine glycol, 5-hydroxymethyl-2'-

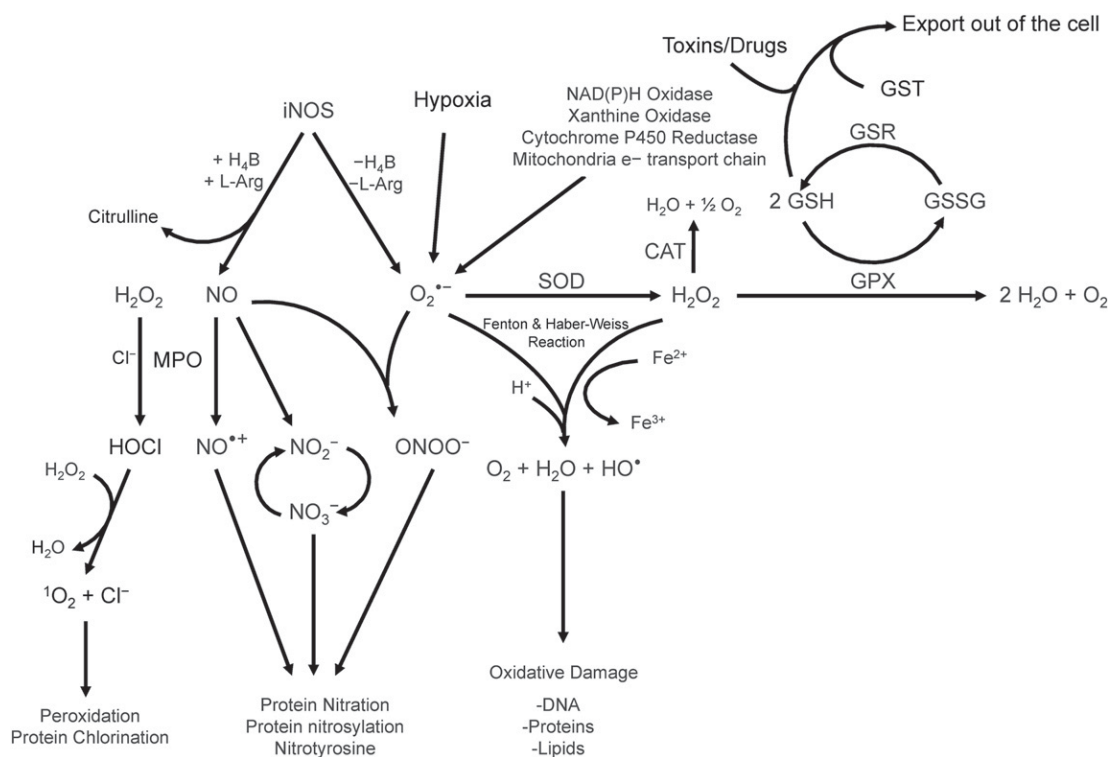


Fig. 1. Summary of key oxidant and antioxidants in cancer. Abbreviations are iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; H_2O_2 , hydrogen peroxide; NO_2^- , nitrite; NO_3^- , nitrate; ONOO^- , peroxynitrite; L-Arg, L-arginine; HO^\bullet , hydroxyl radical; SOD, superoxide dismutase, CAT, catalase; GSH, glutathione; GPX, glutathione peroxidase; GSR, glutathione reductase; GST, glutathione S-transferase; GSSG, reduced glutathione; NAD(P)H, nicotinamide adenine dinucleotide phosphate; H_4B , tetrahydrobiopterin; Fe^{2+} , Iron (II); Fe^{3+} , Iron (III); $\text{O}_2^{\bullet-}$, superoxide; $\text{NO}^{\bullet+}$, nitrosonium cation; Cl^- , chloride ion; HOCl, hypochlorous acid.

deoxyuridine, and 8-OHdG are now considered as markers of DNA damage by oxidative stress [19]. More importantly, ROS are considered an essential factor in the maintenance of the oncogenic phenotype by activation of certain signaling pathways, specifically, the MAPK/AP-1 and NF- κ B pathways [20]. On the other hand, ROS are also required for the induction of cell death and thus can act as antitumor agents, which in this case is dependent on the concentration of ROS in the cellular environment [21].

Additionally, ROS are known to enhance tumor invasion and metastasis by increasing the rates of cell migration [1,11]. The NAD(P)H oxidase family of enzymes, a major source of cellular ROS, has been linked to the promotion of tumor cell survival and growth in pancreatic and lung cancers [1,11]. Reactive oxygen species regulate the expression of intercellular adhesion protein-1 (ICAM-1), a cell surface protein in endothelial and epithelial cells, through the activation of NF- κ B. ICAM-1 and IL-8 regulate the migration of neutrophils across the endothelium, which aid in tumor metastasis [11]. Another key player in the tumor invasion process is the upregulation of specific matrix metalloproteinases (MMPs), such as MMP-2, MMP-3, MMP-9, MMP-10, and MMP-13 by H_2O_2 and NO [11]. The mechanism of MMP upregulation involves the activation of Ras, the MAPK family members ERK1/2, p38, and JNK, or the inactivation of phosphatases [11,22]. Matrix metalloproteinases are essential enzymes in the degradation of most components of the basement membrane and extracellular matrix, such as type IV collagen [11,22].

Angiogenesis is critical for the survival of solid tumors and is also regulated by ROS [11]. Angiogenesis is regulated by a number of oncogenes and tumor-suppressor genes such as Ras, c-Myc, c-Jun, mutated p53, human epidermal growth factor receptor-2, and steroid receptor coactivators through the up-regulation of VEGF or the down-regulation of thrombospondin-1 (TSP-1), an angiogenesis suppressor [11]. Reactive oxygen species stabilizes HIF-1 α protein and induces the production of angiogenic factors by tumor cells.

4. Cancer cells are under intrinsic oxidative stress

Cancer cells are known to manifest increased aerobic glycolysis (Warburg effect) and high levels of intrinsic oxidative stress [23,24]. Hypoxia triggers several critical adaptations that enable cell survival: it suppresses apoptosis, alters glucose metabolism, and triggers an angiogenic phenotype [15,23]. Recent investigations suggest that O_2 depletion stimulates mitochondria to produce ROS, which subsequently activates signaling pathways, such as HIF-1 α , that promote cell survival and consequently, fibrotic growth [15]. Although HIF-1 α is constitutively expressed, its half-life is extremely short because it is rapidly hydroxylated by dioxygen, oxaloglutarate, and iron-dependent prolyl 4-hydroxylases (PHD 1, 2, and 3), located in the nucleus, cytoplasm, or both, respectively [24,25]. Recent studies suggest that NO and ROS, some of which may be of mitochondrial origin, can promote HIF-1 α stabilization by inhibiting (prolyl hydroxylase) PHD activity [15,26]. Superoxide is converted to H_2O_2 by SOD, and the resulting H_2O_2 efflux into the cytosol inhibits PHD activity, allowing HIF-1 α to accumulate, dimerize with HIF-1 β , and translocate into the nucleus where it modulates the expression of genes that favor survival under hypoxic conditions [15]. Support for the role of mitochondrial ROS in HIF-1 α stabilization comes from studies showing that HIF-1 α stabilization can be blocked under hypoxic conditions if ROS production is abrogated in mitochondria that lack cytochrome c or that have been treated with small interfering RNA (siRNA) to knock down the Rieske protein [15,27].

Several pro-oxidant enzymes such as myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and NAD(P)H oxidase have been found in numerous types of malignant tumors including breast, lung, prostate, bladder, colorectal and malignant melanoma, while the expression strongly depends on the histological type/grade of the tumor [9,28,29]. Similarly, antioxidants have also been associated with cancer. Both GSR and GPX expression have been reported to be differentially expressed in various types of cancer [9]. Additionally, CAT was

decreased in breast, bladder, and lung cancer while increased in brain cancer [9,28,29]. Superoxide dismutase is expressed in lung, colorectal, gastric, ovarian, and breast cancer, while decreased activity and expression have been reported in colorectal carcinomas and pancreatic cancer cells [9,28,29]. Collectively, this differential expression of oxidants and antioxidants demonstrates how the microenvironment of cancer is both unique and complex.

5. Ovarian cancer cells manifest a persistent pro-oxidant state

Oxidative stress has been implicated in the pathogenesis of several malignancies, including ovarian cancer [24,30]. Evidence suggests that ovarian cancer patients have decreased levels of circulating antioxidants and higher levels of oxidative stress [10,23,24,30–32]. In the past two decades, it has been reported that epithelial ovarian cancer (EOC) tissues and cells manifest a pro-oxidant state characterized by an increased expression of key pro-oxidant enzymes and decreased expression of antioxidant enzymes [31–33] (Table 1). Specifically, EOC cells and tissues manifested an increased expression of iNOS, MPO, NAD(P)H oxidase, as well as an increase in NO levels which correlated with expression in iNOS [31–33] (Table 1). Moreover, EOC cells manifested lower apoptosis, which was markedly induced by inhibiting iNOS with L-NAME, indicating a strong link between apoptosis and the NO/iNOS pathways in these cells [33]. More importantly, it was found that EOC cells manifested a significant increase in S-nitrosylation of caspase-3, which correlated with a significant decrease in caspase-3 activity, suggesting a potential mechanism of delayed apoptosis that was observed in these cells. Myeloperoxidase is a key oxidant enzyme that utilizes NO produced by iNOS, as a one-electron substrate generating nitrosonium cation (NO^+), a labile nitrosating species [32,34,35]. Interestingly, MPO was only recently found to be expressed by EOC cells and tissues, and has since been confirmed by other investigators [10, 32,36]. Collectively, these findings suggests that MPO is a key player in regulating apoptosis in EOC cells, but also highlights a possible cross talk between iNOS and MPO in ovarian cancer [32].

Myeloperoxidase, an abundant hemoprotein previously known to be present solely in neutrophils and monocytes, plays an essential role in immune surveillance and host defense mechanisms, and can contribute to 3-nitrotyrosine formations in vivo and directly modulates inflammatory responses via regulation of NO bioavailability during inflammation [32,37]. Silencing MPO gene expression utilizing MPO specific siRNA induced apoptosis in EOC cells through a mechanism that involved the S-nitrosylation of caspase-3 by MPO [32]. Additionally, MPO can serve as a source of free iron under oxidative stress, where both NO and $\text{O}_2^{\bullet-}$ are elevated [10,32]. Iron reacts with H_2O_2 and catalyzes the generation of highly reactive hydroxyl radical (HO^\bullet), thereby increasing oxidative stress, which in turn increases free iron concentrations by the Fenton and Haber–Weiss reaction [10,32]. The potential

benefits of the combination of serum MPO and free iron as biomarkers for early detection of ovarian cancer have now been established [10]. Collectively, there is now substantial evidence demonstrating that altered oxidative stress may play a role in maintaining the oncogenic phenotype of ovarian cancer cells, and is summarized in Fig. 2.

6. Oxidative stress triggers cancer cells to favor anaerobic metabolism

Oxidative stress triggers cancer cells to favor anaerobic metabolism, despite the fact that oxygen is present [38,39]. This altered metabolism consists of an increase in glycolysis that is maintained in conditions of high oxygen tension (“aerobic glycolysis”) and gives rise to enhanced lactate production [38–40]. To compensate for the reduction in cellular ATP production, [aerobic glucose oxidation generates more ATP per glucose molecule (36 ATP) as compared to glycolysis (2 ATP)], and cancer cells upregulate glucose receptors and significantly increase glucose uptake [24,25,40]. Aerobic glycolysis, in tumor cells, results in significant lactic acidosis, which additionally induces substantial toxicity to the surrounding tissues and in cancer cells themselves. Furthermore it has been shown that lactic acidosis facilitates tumor growth, in part through breakdown of extracellular matrix, increased cell mobility/metastatic potential, and activation of angiogenesis [40]. One of the foremost nearly ubiquitous mechanisms of aerobic glycolysis resides in the activation of HIF, an oxygen-sensitive transcription factor that is activated by hypoxic stress as well as oncogenic, inflammatory, metabolic, and oxidative stress [40]. The link between oxidative stress and aerobic glycolysis is supported by the fact that HIF is activated under hypoxic conditions and is known to induce the expression of several glucose transporters as well as most of the enzymes required for glycolysis [41]. Hypoxia-inducible factor also induces the expression of pyruvate dehydrogenase kinase (PDK), an enzyme that regulates the entry of pyruvate into the mitochondria [25,40,42]. Activated PDK can inhibit pyruvate dehydrogenase (PDH), thereby limiting the entry of pyruvate into the mitochondria, where glucose oxidation can occur.

Dichloroacetate (DCA) is a metabolic modulator that has been clinically utilized in the treatment of hereditary mitochondrial diseases as well as lactic acidosis [25,43]. Dichloroacetate inhibits PDK and thus shifts glucose metabolism in cancer cells from glycolysis to glucose oxidation, reversing the unique aerobic glycolysis found in solid tumors [44]. Consistent with these findings, DCA treatment significantly decreased HIF-1 α expression [24]. Dichloroacetate has been shown to shift the oxidative balance in the intracellular redox state, leading to the activation of specific endonucleases, which induce apoptosis in EOC cells [24]. Treatment of EOC cells with DCA significantly induced apoptosis through the stimulation of caspase-3 activity in a dose-dependent manner, and was confirmed by the TUNEL assay [24]. Indeed, DCA has also been shown to induce apoptosis in cancer cells as evident by the efflux of cytochrome *c* and apoptosis-inducing factor from the mitochondria [45]. In support of these findings, it has been shown that aerobic glycolysis, as a result of oxidative stress, can result in resistance to apoptosis [24,46]. Several enzymes involved in glycolysis are also known to regulate apoptosis and gene transcription, suggesting that links between metabolic sensors, cell death, and gene transcription are established directly through the enzymes that control metabolism [25, 47]. Additionally, DCA induces apoptosis in glioblastoma, endometrial, prostate, and nonsmall cell lung cancers, further supporting the findings from this study, which aimed to establish a link between DCA, oxidative stress, and apoptosis in EOC cell lines, possibly through similar mechanisms [25].

Since DCA acts by activating PDH, through the inhibition of PDK, bringing pyruvate into the mitochondria and enhancing glucose oxidation, it is therefore an ideal approach to shift aerobic glycolysis to glucose oxidation coupling rather than just inhibiting aerobic glycolysis. Inhibiting aerobic glycolysis results in ATP depletion and necrosis, not apoptosis, because apoptosis is an energy-consuming process, requiring

Table 1

Summary of oxidant and antioxidant expression in sensitive and chemoresistant ovarian cancer. Abbreviations are iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; SOD, superoxide dismutase, CAT, catalase; GSH, glutathione; GPX, glutathione peroxidase; GSR, glutathione reductase; NAD(P)H, nicotinamide adenine dinucleotide phosphate.

	Ovarian cancer	Chemoresistant ovarian cancer	Reference
<i>Oxidants</i>			
MPO	↑	↑↑	[10,32,36]
iNOS	↑	↑↑	[28,32]
Nitrite/nitrate		↑↑	[9,28]
NAD(P)H oxidase	↑		[31]
<i>Antioxidants</i>			
CAT	↓	↑↑	[9,30]
GSH	↑↑	↑↑	[58]
GSR		↓↓	[9,28]
GPX		↑↑	[9]
SOD	↓	↓↓	[9,30]

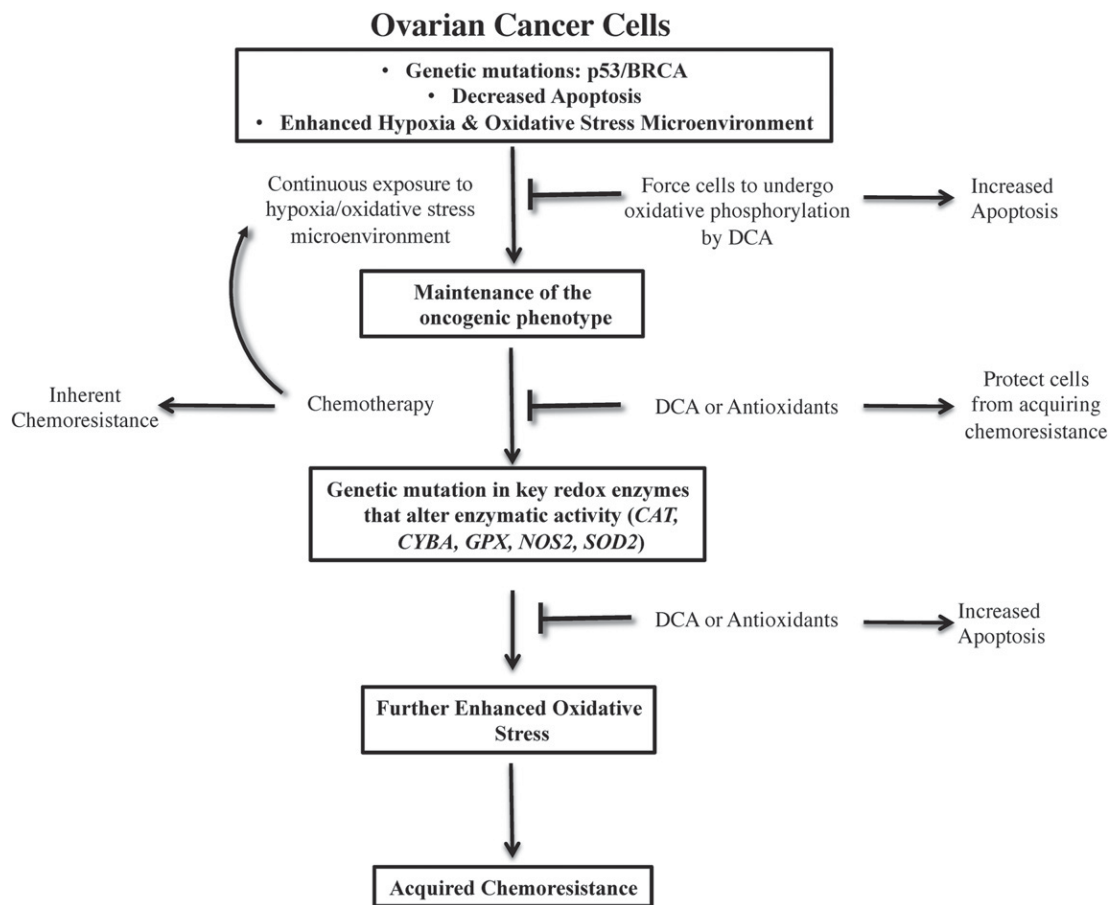


Fig. 2. Summary of the role of oxidative stress in the development of sensitive and chemoresistant ovarian cancer.

active mitochondria [25,48]. Dichloroacetate activates PDH through the inhibition of PDK at concentrations of 10 to 250 mmol/L or 0.15 to 37.5 mg/mL, in a dose-dependent fashion [25,49]. Four different isoforms of PDK have been identified that have variable expression and sensitivity to the inhibition by DCA [25,50]. Moreover, DCA administered at 35 to 50 mg/kg decreases lactate levels by more than 60% and directly activates PDH by 3- to 6-fold [25,49].

The high levels of ROS and RNS manifested by tumor cells can be countered by high levels of antioxidants, such as SOD [51]. Superoxide dismutase is considered a key antioxidant in aerobic cells and is responsible for the elimination of $O_2^{\bullet-}$ by converting it to H_2O_2 . Indeed, deficiency in SOD or inhibition of the enzyme activity may cause accumulation of $O_2^{\bullet-}$ in the cells, which may result in the persistence of the oncogenic phenotype [52]. Interestingly, DCA has been shown to significantly induce the expression of SOD3 in EOC cells, however, in other studies using different cancer cell lines, it was reported that decreased levels of SOD are effective in the induction of apoptosis [23,24,53]. Decreased levels of SOD may result in toxic high levels of free radicals, which ultimately could lead to necrosis. On the other hand, ROS can also induce cellular senescence and cell death and can therefore function as antitumorigenic agents [24,54]. Whether ROS promote tumor cell survival or act as antitumorigenic agents depends on the cell and tissues, the location of ROS production, and the concentration of individual ROS [11].

In summary, studies have shown that shifting anaerobic to aerobic metabolism by DCA induces apoptosis of EOC cells [24]. This effect was attributed to the modulation of key enzymes that are central to controlling the cellular redox balance. The utilization of DCA to induce apoptosis of EOC cells may provide a therapeutic option in the treatment of EOC. Explicitly, the potential therapeutic value of DCA for ovarian cancer will require future analysis utilizing more cell lines, including

ovarian surface epithelial cells, fallopian tube secretory epithelial cells, as well as patients.

7. Chemotherapy and the acquisition of chemoresistance in EOC cells

Despite significant advances in surgery and anticancer treatment, chemotherapy resistance remains a major obstacle to improving a cancer patient's outcome [55]. Taxanes and platinum are the current drug therapies used for treatment of ovarian cancer. Chemoresistance greatly limits the range of possibilities for subsequent treatments, because some tumors become resistant not only to the initial drug but also to new therapeutic agents with different mechanisms of action [56]. Many chemotherapy drugs serve as a source of oxidative stress through a direct mechanism of cell death, or as an indirect effect of exposure, as observed with several chemotherapeutic agents [57]. Known factors affecting the occurrence of resistance include: altered drug influx/efflux, increased cellular GSH levels, upregulation of *Bcl-2*, decreased platinum accumulation in tumor cells, increased GSH synthesis, loss of tumor necrosis factor receptor apoptosis-inducing ligand (TRAIL)-induced apoptosis, increased DNA repair and enhanced ability to repair through up-regulation of DNA repair genes [11]. Moreover, overexpression of GST is known to reduce the reactivity of various chemotherapy drugs [58]. Additionally, loss of functional p53 augments NF- κ B activated-inflammation, thus, stabilization of wild-type p53 is critical for the prevention of EOC from progression to drug-resistance [11]. Chemoresistant EOC cells have been shown to exhibit increased expression of iNOS and nitrate/nitrite levels as well as a decrease in GSR expression, suggesting a shift towards a severe pro-oxidant state by these cells [28] (Table 1).

As mentioned earlier, EOC cells are known to manifest a pro-oxidant state characterized by increased key oxidant enzymes with concomitant decreased antioxidant enzymes [28] (Table 1). Chemotherapy resistant EOC cells are now known to also manifest an alteration in the redox balance, further advancing this pro-oxidant environment [9]. Indeed, there was a significant increase in levels of CAT, GPX, and iNOS in chemoresistant EOC cells as compared to their sensitive counterparts [9] (Table 1). In contrast, there was a decrease in levels in GSR, SOD, and the NAD(P)H oxidase subunit (p22^{phox}) in chemoresistant EOC cells [9]. This data supports an important role for an altered redox balance, not only in the maintenance of the oncogenic phenotype, but also in the development of chemoresistance (Fig. 2).

8. Polymorphisms in key oxidant and antioxidant enzymes are associated with ovarian cancer

A single nucleotide polymorphism (SNP) occurs because of point mutations that are selectively maintained in populations and are distributed throughout the human genome at an estimated overall frequency of at least one in every 1000 base pairs [59]. Recent evidence demonstrates an association between enzymatic activity altering SNPs in oxidative DNA repair genes and antioxidant genes with human cancer susceptibility [13]. Additionally, a pro-oxidant state has been implicated in the pathogenesis of several malignancies, including ovarian cancer [24,31]. This area of research is essentially reorganizing our understanding of inheritance and evolution. These modifications might explain the in vitro persistence of the oncogenic phenotype even after normal conditions are restored, as well as the clinical propensity for individuals to develop cancer.

This mechanism of altered enzymatic activity further explains the observation of significantly decreased apoptosis and increased survival of EOC cells [32]. Investigations into the effect of SNPs on various redox enzymes are an active area of scientific research [9,29,60,61]. The effects of genetic polymorphisms in oxidative stress-related genes on cancer susceptibility may be determined by studying functional polymorphisms in genes that control the levels of cellular ROS and oxidative damage, including SNPs for genes involved in carcinogen metabolism (detoxification and/or activation), antioxidants, and DNA repair pathways [60]. Several SNPs have been identified in key antioxidants, leading to change of function, including CAT, GPX1, GSR, and SOD2 [9, 61]. In support of this, recent studies have also associated genetic polymorphisms in genes involved in suppression of tumorigenicity as well as those involved in cell cycle with ovarian cancer [62,63]. Additional genetic variations, many of which have been identified in recent genome-wide association studies (GWAS), have been hypothesized to act as low to moderate penetrant alleles, which contribute to ovarian cancer risk, as well as other diseases [7,64].

There now is convincing evidence to suggest an association of specific SNPs in key redox enzymes with increased risk and overall survival of ovarian cancer [9,29]. Recently, a specific CAT SNP (rs1001179), that leads to reduced enzyme activity, was reported to be associated with increased risk for breast cancer and has also been described to be a significant predictor of death when present in ovarian cancer patients [9,29, 61,65]. This finding is consistent with several other studies, which linked this specific SNP with risk, response to adjuvant treatment and survival of cancer patients, including ovarian [29,66].

NAD(P)H oxidase, a key pro-oxidant enzyme, is a significant source of ROS. The membrane bound components of NAD(P)H oxidase are the catalytic subunit CYBB (gp91^{phox}) and the adjacent oxygen sensing subunit CYBA (p22^{phox}) [9,29]. Several SNPs for CYBA have been reported, some of which alter the enzyme activity. A specific SNP in CYBA (rs4673) was associated with an increased risk for ovarian cancer and other diseases where oxidative stress plays a critical role in their pathophysiology, including cardiovascular disease, asthma, and diabetic nephropathy [9,29]. The mutant genotype of the CYBA gene has been

shown to both decrease and increase activity of the protein, thereby altering the generation of O₂•⁻ [9,29].

Recent genetic studies have linked MPO to lung and ovarian cancers by demonstrating a striking correlation between the relative risk for development of the disease and the incidence of functionally distinct MPO polymorphisms [9,29]. Specifically, a SNP in MPO (rs2333227) was shown to be associated with increased risk for ovarian cancer [36]. Genome-wide association studies have also successfully identified and confirmed six SNPs that appear to influence the risk of EOC [9,29]. The confirmed susceptibility SNPs are rs3814113 (located at 9p22, near BNC2), rs2072590 (located at 2q31, which contains a family of HOX genes), rs2665390 (located at 3q25, intronic to TIPARP), rs10088218 (located at 8q24, 700 kb downstream of MYC), rs8170 (located at 19p13, near MERIT40), and rs9303542 (located at 17q21, intronic to SKAP1) [9,29]. Therefore, some believe that the genetic component of ovarian cancer risk may be attributed to genetic polymorphisms that confer low to moderate risk, such as SNPs that result in point mutations in the gene [67].

9. Acquisition of chemoresistance in ovarian cancer cells is associated with specific point mutations in key redox enzymes

The mechanisms underlying the acquisition of chemoresistance in ovarian cancer have yet to be fully elucidated. Evidence for an enhanced pro-oxidant state in chemoresistant EOC cells has now been described, and is thought to be a result of point mutations in key redox enzymes [9]. Specifically, a recent study observed a significant increase in levels of CAT, GPX, and iNOS while there was a significant decrease in levels of GSR, SOD, and NAD(P)H oxidase in chemoresistant EOC cells as compared to their sensitive counterparts [9]. These findings suggest a role for an altered redox balance in the development of chemoresistance in ovarian cancer. To investigate a possible mechanism of altered redox enzyme levels, the presence of several SNPs was determined in both sensitive and chemoresistant EOC cell lines. Indeed, docetaxel and/or cisplatin chemoresistant EOC cells were characterized to manifest specific point mutations, corresponding to known functional SNPs, in key redox enzymes including SOD2 (rs4880), NOS2 (rs2297518), and CYBA (rs4673) which are not present in their sensitive counterparts (Table 1). Interestingly, chemoresistant EOC cells exhibited an altered enzymatic activity for CAT and GSR while they did not exhibit the specific SNP of interest in those enzymes, which again suggests possible involvement in other functional SNPs for those enzymes (Table 1) [9]. The fact that the SNP was present in the chemoresistant EOC cells and not the sensitive cell line from which it was derived suggests that in fact, this is a point mutation rather than a SNP. To determine whether chemotherapy was capable of inducing point mutations that happen to correspond to known functional SNPs, specific point mutations in SOD2 or GPX1 were induced in sensitive EOC cells which led to a decrease in the sensitivity to chemotherapy, suggesting acquisition of chemoresistance [9]. Furthermore, treatment of sensitive and chemoresistant EOC cells with SOD combined with chemotherapy significantly increased the efficacy of the chemotherapy in a synergistic manner, with a more drastic effect in the chemoresistant cells [9]. This observation suggests that induction of specific point mutations in sensitive EOC cells corresponding to functional SNPs found in chemoresistant EOC cells directly reduced the sensitivity to chemotherapy (Fig. 2). These findings also support the notion that chemotherapy can induce gene point mutations that happen to correspond to SNPs in locations with functional effects, thus altering overall redox balance for survival (Fig. 2) [9].

One possible explanation for the observed nucleotide switches in response to chemotherapy is nucleotide substitution, a mechanism which includes transitions, replacement of one purine by the other or that of one pyrimidine by the other, or transversions, replacement of a purine by a pyrimidine or vice versa [9]. It has been established that hydroxyl radicals react with DNA causing the formation of a large number of

pyrimidine and purine-derived lesions [9]. The oxidative damage to 8-Oxo-2'-deoxyguanosine, an oxidized derivative of deoxyguanosine and major product of DNA oxidation, has been implicated in tumor initiation and progression through accumulation of genetic alterations of both oncogenes and tumor suppressor genes [9]. Indeed, previous findings revealed that GC → TA transversions derived from 8-hydroxy-2'-deoxyguanosine have been reported in the *ras* oncogene and the *p53* tumor suppressor gene in several cancers. It should be indicated however that GC → TA transversions are not unique to hydroxy-2'-deoxyguanosine, CC → TT substitutions have been identified as signature mutations for ROS [9].

Another explanation for the nucleotide switch is that chemoresistance resulted in an entirely different population of cells, with a new genotype. Chemotherapy eliminates the bulk of the tumor while leaving a core of cancer cells with high capacity for repair and renewal, known as cancer stem cells (CSCs) [9]. Tumors arising from CSCs usually contain a mixed population of cells due to the property of asymmetric division [9]. Cancer stem cells have been isolated from various types of cancer including leukemia, breast, brain, pancreatic, prostate, ovarian and colon [9]. Strikingly, CSCs were reported to be present in SKOV-3 EOC cells [9]. Additionally, CSCs have been shown to confer chemoresistance to cisplatin and doxorubicin in ovarian cancer cells [9].

10. Ovarian cancer immunotherapy and oxidative stress

It is well established that tumorigenic cells generate high levels of ROS to activate proximal signaling pathways that promote proliferation, survival and metabolic adaptation while also maintaining a high level of antioxidant activity to prevent buildup of ROS to levels that could induce cell death [68]. Moreover, there is evidence that ROS can act as second messengers in immune cells, which can lead to hyperactivation of inflammatory responses resulting in tissue damage and pathology [68]. Ovarian cancer is considered an ideal tumorigenic cancer because ovarian cancer cells have no negative impact on immune cells [69].

Effective immunotherapy for ovarian cancer is currently the focus of several investigations and clinical trials. Current immunotherapies for cancer treatment include therapeutic vaccines, cytokines, immune modulators, immune checkpoint inhibitors, and adoptive T cell transfer [70]. The discovery of a monoclonal antibody (bevacizumab) directed against vascular endothelial growth factor (VEGF) which has been shown to improve progression free survival compared to cytotoxic chemotherapy alone was a major outcome of clinical trials [71]. Other monoclonal antibodies currently approved for other cancers such as trastuzumab for breast cancer or cetuximab for colon cancer exhibited limited activity in ovarian cancer [71]. Several clinical trials are ongoing for the utilization of immune checkpoint blockade in ovarian cancer immune therapy [72]. Most recently tested were the programmed death (PD)-1 inhibitors, pembrolizumab and nivolumab, which showed a consistent response rate of 10–20% in phase 2 studies and then failed to improve outcomes in confirmatory trials [72]. Ultimately, larger phase 3 studies are needed to validate these findings for checkpoint inhibitors, particularly with regard to the duration of response seen with these agents. Additionally, the direct intraperitoneal delivery of interleukin (IL)-12, a potent immunostimulatory agent, exhibited some potential therapeutic efficacy in ovarian cancer [73]. Recently, targeting folate receptor alpha, which is found to be expressed in ovarian cancer, has shown promising therapeutic value. The targeting of the folate receptor was achieved by either a blocking monoclonal antibody (farletuzumab) or antibody conjugates of folate analogs, such as vintafolide [74].

11. Summary and conclusion

Oxidative stress has been implicated in the pathogenesis of several malignancies including ovarian cancer. Epithelial ovarian cancer is characterized to manifest a persistent pro-oxidant state through alteration

of the redox balance, which is further enhanced in their chemoresistant counterparts, as summarized in Table 1 and Fig. 2. Forcing ovarian cancer cells to undergo oxidative phosphorylation rather than glycolysis has been shown to be beneficial for eliminating cells via apoptosis (Fig. 2). Collectively, there is convincing evidence that indicated a causal relationship between the acquisition of chemoresistance and chemotherapy-induced genetic mutations in key redox enzymes, leading to a further enhanced oxidative stress in chemoresistant EOC cells. This concept was further confirmed by the observation that induction of point mutations in sensitive EOC cells increased their resistance to chemotherapy. Also, a combination of antioxidants with chemotherapy significantly sensitized cells to chemotherapy. Identification of targets for chemoresistance with either biomarker and/or screening potential will have a significant impact for the treatment of this disease.

Conflicts of interest

GMS and NMK disclose no potential conflicts of interest. MPD receives grant and contract support from the NIH/NICHD, Abbvie, Bayer, and PCORI/AHRQ. MPD is also a stockholder and on the Board of Directors for Advanced Reproductive Care, LLC.

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IN THE UNITED STATES DISTRICT COURT
FOR THE EASTERN DISTRICT OF NEW JERSEY

IN RE: JOHNSON & JOHNSON
TALCUM POWDER PRODUCTS
MARKETING, SALES PRACTICES,
AND PRODUCTS LIABILITY
LITIGATION

THIS DOCUMENT RELATES TO
ALL CASES

Case No. 16-2738
(FLW) (LHG)

MDL Docket No. 2738

Friday, March 29, 2019

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The video deposition of MICHAEL BIRRER, M.D.,
Ph.D., taken pursuant to notice, was held at
the law offices of Butler Snow, LLP, One Federal
Place, Suite 1000, 1819 Fifth Avenue North,
Birmingham, Alabama, commencing at approximately
9:03 a.m., on the above date, before Lois Anne
Robinson, Registered Diplomate Reporter,
Certified Realtime Reporter, and
Notary Public for the State of Alabama.

Michael Birrer, M.D., Ph.D.

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<p>1 APPEARANCES</p> <p>2 COUNSEL FOR PLAINTIFFS' STEERING COMMITTEE:</p> <p>3 BEASLEY ALLEN LAW FIRM</p> <p>4 218 Commerce Street</p> <p>5 Montgomery, Alabama 36104</p> <p>6 BY: Margaret M. Thompson,</p> <p>7 M.D., J.D., MPAFF</p> <p>8 Margaret.thompson@beasleyallen.com</p> <p>9 Sydney Everett, Esquire</p> <p>10 Sydney.everett@beasleyallen.com</p> <p>11</p> <p>12 ROBINSON CALCAGNIE, INC.</p> <p>13 19 Corporate Plaza Drive</p> <p>14 Newport Beach, California 92660</p> <p>15 BY: Cynthia L. Garber, Esquire</p> <p>16 Cgarber@robinsonfirm.com</p> <p>17</p> <p>18 RESTAINO LAW, LLC</p> <p>19 130 Forest Street</p> <p>20 Denver, Colorado 80220</p> <p>21 BY: John M. Restaino, Jr., DPM, ESQ.</p> <p>22 Jrestaino@restainollc.com</p> <p>23</p> <p>24 NAPOLI SHKOLNIK PLLC</p> <p>400 Broadway Road, Suite 305</p> <p>Melville, New York 11747</p> <p>BY: ALASTAIR J. M. FINDEIS, ESQUIRE</p> <p>Afindeis@napolilaw.com</p> <p>FOR THE DEFENDANT, JOHNSON & JOHNSON:</p> <p>NUTTER, McCLENNEN & FISH, LLP</p> <p>155 Seaport Boulevard</p> <p>Boston, Massachusetts 02210</p> <p>BY: Dawn M. Curry, ESQUIRE</p> <p>Dawn@nutter.com</p> <p>SKADDEN, ARPS, SLATE, MEAGHER & FLOM, LLP</p> <p>4 Times Square</p> <p>New York, New York 10036</p> <p>BY: Benjamin Halperin, Esquire</p> <p>Benjamin.halperin@skadden.com</p>	<p>1 INDEX</p> <p>2 EXAMINATION PAGE</p> <p>3 By Ms. Thompson 10</p> <p>4 By Ms. Curry 424</p> <p>5</p> <p>6 * * * * *</p> <p>7 EXHIBITS</p> <p>8 1 Expert report of Michael Birrer, M.D., Ph.D. 20</p> <p>9</p> <p>10 2 Curriculum vitae of Michael Birrer, M.D., Ph.D. 21</p> <p>11</p> <p>12 3 Defendants' Response to Plaintiff's Document 21</p> <p>13 Requests contained in Notice</p> <p>14</p> <p>15 4 Health Canada - Draft Screening Assessment - Talc 48</p> <p>16</p> <p>17 5 FDA letter to Samuel Epstein regarding Citizen 49</p> <p>18 Petitions</p> <p>19</p> <p>20 6 IARC Monographs on the Evaluation of Carcinogenic 50</p> <p>21 Risks to Humans</p> <p>22</p> <p>23 7 "Weight of Evidence: General Principles and 53</p> <p>24 Current Applications at Health Canada"</p>
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<p>1 VIDEOGRAPHER: 2 We are now on the record. My name is 3 Devyn Mulholland. I'm a videographer for Golkow 4 Litigation Services. Today's date is March 29th, 5 2019. The time is 9:03 a.m. 6 This video deposition is being held in 7 Birmingham, Alabama, in the matter of Talcum 8 Powder Litigation, MDL Number 2738. The deponent 9 is Michael Birrer, M.D., Ph.D. 10 Counsel will be noted on the 11 stenographic record. The court reporter is Lois 12 Robinson and will now swear in the witness. 13 MICHAEL BIRRER, M.D., PH.D., 14 the witness, after having first been 15 duly sworn to tell the truth, the whole truth, 16 and nothing but the truth, was examined and 17 testified as follows: 18 EXAMINATION 19 BY MS. THOMPSON: 20 Q Dr. Birrer, I'm Margaret Thompson, and 21 I'll be taking your deposition today. 22 You've had your deposition taken 23 before; right? 24 A Correct.</p>	<p>1 It -- it eventually went to -- to court. They 2 have a panel up there of three judges, which sort 3 of prescreens it. 4 Q And you've also submitted a previous 5 report in this case; correct? 6 MS. CURRY: 7 Object to the form. 8 A Correct. 9 MS. THOMPSON: 10 Q That was in the Swan case? Does that 11 sound familiar? 12 A Yes. 13 Q Have any of your opinions -- and that 14 was in May 2017. Does that sound right? 15 A That sounds right. 16 Q Have any of your opinions in this case 17 changed since May 2017? 18 A No. 19 Q Have any of your opinions changed since 20 you were deposed in September of 2018? 21 A No. 22 Q I guess that would be a "no" if they 23 hadn't changed since 2017. 24 A It's consistent.</p>
Page 11	Page 13
<p>1 Q Including in the talcum powder 2 litigation; correct? 3 A Yes. 4 Q Have you had your deposition taken in 5 any other situation? 6 A I gave testimony in a case, but that 7 wasn't a deposition, I don't think. No. 8 Q And when was that? 9 A That was prior to the talc. It's -- 10 probably goes back, I want to say, 2015, 2012, 11 somewhere -- 12 Q And what -- sorry. 13 A Yeah. 14 Q What was the nature of that matter? 15 A I was in Massachusetts at the time. It 16 was a delayed diagnosis case. 17 Q A medical malpractice case? 18 A Medical malpractice, yes. 19 Q Were you testifying for the plaintiff 20 or for the defendant? 21 A Defendant. 22 Q Was it a physician or a doc- -- a 23 hospital? 24 A It was both. And it was in Maine.</p>	<p>1 Q And you're aware that the purpose of 2 today is for me to gain a thorough understanding 3 of what opinions you plan to give at a hearing or 4 trial? 5 A Yes. 6 Q And the basis for those opinions; 7 right? 8 A Yes. 9 Q And your report states that your 10 opinions are given to a reasonable degree of 11 scientific and medical certainty. 12 What does that mean to you? 13 A It means that, basically, more often 14 than not, they're correct. 15 Q And you are a medical doctor as well as 16 a Ph.D. researcher; correct? 17 A Correct. 18 Q Do you currently see patients? 19 A I do. 20 Q Do you currently diagnose ovarian 21 cancer in women? 22 A Yes. 23 Q How -- do you treat women with ovarian 24 cancer?</p>

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<p style="text-align: right;">Page 14</p> <p>1 A Yes.</p> <p>2 Q And would that primarily involve the</p> <p>3 medical aspects, including chemotherapy</p> <p>4 administration?</p> <p>5 A Yes.</p> <p>6 Q Do you perform any surgical procedures?</p> <p>7 A No.</p> <p>8 Q What --</p> <p>9 A I'm a medical oncologist.</p> <p>10 Q What --</p> <p>11 A I could perform it, but it wouldn't</p> <p>12 come out very well.</p> <p>13 Q I understand.</p> <p>14 What percentage of your time involves</p> <p>15 patient care versus research?</p> <p>16 A So --</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A -- right now I have a half-a-day clinic</p> <p>20 a week, and then the research component, I have a</p> <p>21 fully funded lab, probably two days a week. I'm</p> <p>22 the director of the cancer center, which also</p> <p>23 takes a fair amount of administrative</p> <p>24 responsibility.</p>	<p style="text-align: right;">Page 16</p> <p>1 A Yes.</p> <p>2 Q And does that pretty much cover the</p> <p>3 types of research that you would be doing in your</p> <p>4 lab --</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 MS. THOMPSON:</p> <p>8 Q -- or in a general sense?</p> <p>9 A I'm just trying to think if there was</p> <p>10 anything else. We obviously do a lot of</p> <p>11 review-type papers and articles. You know, I</p> <p>12 think that's pretty broad. I think it does,</p> <p>13 actually.</p> <p>14 Q When you do a review article, is that</p> <p>15 usually invited by the journal, or is that a</p> <p>16 topic that you have interest in that you submit</p> <p>17 as a publication?</p> <p>18 A Could be both. A lot of them are</p> <p>19 invited. But we have occasionally thought of</p> <p>20 areas that we thought were interesting and</p> <p>21 important and suggested it.</p> <p>22 Q And are authors or review articles</p> <p>23 generally intended to be experts in the field?</p> <p>24 MS. CURRY:</p>
<p style="text-align: right;">Page 15</p> <p>1 MS. THOMPSON:</p> <p>2 Q So administrative time --</p> <p>3 A Yeah.</p> <p>4 Q -- as well included in that?</p> <p>5 And how would you describe the focus of</p> <p>6 your laboratory search -- research currently?</p> <p>7 A Almost entirely on ovarian cancer and</p> <p>8 exploring detailing the genomics, the molecular</p> <p>9 basis for ovarian cancer and trying to translate</p> <p>10 that into better early detection, diagnosis and</p> <p>11 treatment.</p> <p>12 Q Are you doing in vitro as well as in</p> <p>13 vivo research?</p> <p>14 A Correct.</p> <p>15 Q And have published in both animal</p> <p>16 studies as well as cellular studies?</p> <p>17 A Yes.</p> <p>18 Q Have you published with immortalized</p> <p>19 cells?</p> <p>20 A Yes.</p> <p>21 Q Have you published research with human</p> <p>22 tissue?</p> <p>23 A Yes.</p> <p>24 Q Have you published human trials?</p>	<p style="text-align: right;">Page 17</p> <p>1 Object to the form.</p> <p>2 A More often than not, yes. But</p> <p>3 frequently on my reviews, I'll have some junior</p> <p>4 people.</p> <p>5 MS. THOMPSON:</p> <p>6 Q With -- with a senior author</p> <p>7 usually --</p> <p>8 A (Nods affirmatively.)</p> <p>9 Q -- correct?</p> <p>10 A Correct.</p> <p>11 Q And that would be, I would think,</p> <p>12 because readers of a journal want to know that</p> <p>13 it's an expert in the field that's providing the</p> <p>14 information in a review article; right?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A I think so, yeah.</p> <p>18 MS. THOMPSON:</p> <p>19 Q Would you agree with me that it would</p> <p>20 be unethical at this point in time to design a</p> <p>21 prospective study in which women were exposed to</p> <p>22 talcum powder in the genital area and follow over</p> <p>23 time?</p> <p>24 MS. CURRY:</p>

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<p style="text-align: right;">Page 18</p> <p>1 Object to the form.</p> <p>2 A Prospectively and randomized and --</p> <p>3 could you just --</p> <p>4 MS. THOMPSON:</p> <p>5 Q Let's start with just prospectively.</p> <p>6 A I -- I think it would be a --</p> <p>7 interesting question. I don't think it would be</p> <p>8 valuable.</p> <p>9 Q How about a randomized trial? Would it</p> <p>10 be ethical?</p> <p>11 A No. I don't think it would be valuable</p> <p>12 at all.</p> <p>13 Q But I didn't ask about valuable.</p> <p>14 What about ethical?</p> <p>15 A Well, val- -- if it's not valuable, it</p> <p>16 should -- it wouldn't be of great concern to do</p> <p>17 that. I'm not sure what you're asking.</p> <p>18 Q Well, I'm asking if you -- if you have</p> <p>19 a carcinogen, even a possible carcinogen, you</p> <p>20 could not design and get a trial through IRB</p> <p>21 using that product and a control group; correct?</p> <p>22 MR. MIZGALA:</p> <p>23 Object to form.</p> <p>24 A I guess -- I -- I see what -- now I see</p>	<p style="text-align: right;">Page 20</p> <p>1 A And this is -- this is a -- let me get</p> <p>2 my glasses -- supplemental materials received by</p> <p>3 me after this was done.</p> <p>4 Q Okay.</p> <p>5 A Okay?</p> <p>6 Q And, so, "received by" you meant the</p> <p>7 lawyers for Johnson & Johnson provided those</p> <p>8 supplemental materials to you?</p> <p>9 A It was a little bit of both. I mean,</p> <p>10 some of this I wasn't privy to, so I got it</p> <p>11 provided to me, and some of these were additional</p> <p>12 articles that I was -- I pulled out.</p> <p>13 Q Okay. And I've marked as Exhibit 1</p> <p>14 your expert report.</p> <p>15 (DEPOSITION EXHIBIT NUMBER 1</p> <p>16 WAS MARKED FOR IDENTIFICATION.)</p> <p>17 MS. THOMPSON:</p> <p>18 Q Do you --</p> <p>19 Do you have a copy? You're good on</p> <p>20 that?</p> <p>21 A And mine's -- mine's thicker than</p> <p>22 yours, so -- it's got my CV in there.</p> <p>23 Q I separated out your CV. So -- well,</p> <p>24 good. But that's a good observation.</p>
<p style="text-align: right;">Page 19</p> <p>1 what you're asking.</p> <p>2 So my position on that is that talc</p> <p>3 is -- I don't believe talc is a carcinogen.</p> <p>4 MS. THOMPSON:</p> <p>5 Q I understand. But there are others</p> <p>6 that do.</p> <p>7 And, so, is it your opinion that an IRB</p> <p>8 would let a study through using what has been</p> <p>9 designated as a possible carcinogen, say, for</p> <p>10 example, IARC?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A I have no idea.</p> <p>14 MS. THOMPSON:</p> <p>15 Q All right. So the ground rules are</p> <p>16 we'll try not to interrupt each other. Let me</p> <p>17 know if I ask a bad question or one that you</p> <p>18 don't understand, and I'll expect you to answer</p> <p>19 honestly. Fair enough?</p> <p>20 A Yes.</p> <p>21 Q If you need a break, let me know.</p> <p>22 What did you bring with you today?</p> <p>23 A I have my expert report right here.</p> <p>24 Q And is that all you brought with you?</p>	<p style="text-align: right;">Page 21</p> <p>1 And -- and I marked as Exhibit 2 your</p> <p>2 CV.</p> <p>3 A Okay.</p> <p>4 (DEPOSITION EXHIBIT NUMBER 2</p> <p>5 WAS MARKED FOR IDENTIFICATION.)</p> <p>6 MS. THOMPSON:</p> <p>7 Q And that should --</p> <p>8 And you're good on that, too?</p> <p>9 MS. CURRY:</p> <p>10 Thank you.</p> <p>11 MS. THOMPSON:</p> <p>12 Q That should -- those combined should be</p> <p>13 the same thickness of what you've brought.</p> <p>14 And I also brought the Notice of</p> <p>15 Deposition, which I'm going to hand you.</p> <p>16 (DEPOSITION EXHIBIT NUMBER 3</p> <p>17 WAS MARKED FOR IDENTIFICATION.)</p> <p>18 MS. THOMPSON:</p> <p>19 Q And this is the one with objections.</p> <p>20 Have you seen this before, Dr. Birrer?</p> <p>21 A Yes.</p> <p>22 Q And did you look at the request on</p> <p>23 the -- on this document?</p> <p>24 A Yes.</p>

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<p style="text-align: right;">Page 22</p> <p>1 Q Is there -- and there's nothing that 2 was responsive to any of these requests? 3 MS. CURRY: 4 Objection. Subject to the objections 5 that were made by counsel. 6 MS. THOMPSON: 7 Q Subject -- 8 MS. THOMPSON: 9 Sorry. 10 Q Subject to the objections. 11 A Yeah. 12 Q So where would you keep your file for 13 the litigation? 14 MS. CURRY: 15 And I'm sorry. Just to clarify for the 16 record, there is a small production at the back 17 that incorporates the -- 18 MS. THOMPSON: 19 Yes. 20 MS. CURRY: 21 -- invoice as well as the supplemental 22 fee schedule and the supplemental list of 23 materials. 24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 24</p> <p>1 Q -- this litigation? 2 And be careful not to interrupt just 3 because it makes our court reporter's job a 4 little more difficult. 5 How much money have you been paid total 6 by Johnson & Johnson in talcum powder litigation? 7 A To date, nothing. 8 Q You haven't been paid for any of the 9 other cases that you've testified in? 10 A Correct. 11 Q Why is that? 12 A I'm a lousy businessman. I haven't 13 invoiced for Swan yet and I haven't invoiced for 14 Brower. But I can -- I can estimate the hours. 15 Q Go ahead and estimate. 16 A Swan I think is around 80 hours -- 17 Q Okay. 18 A -- because it was the initial case. It 19 was a bundled -- bundled five cases, so involved 20 a lot of review. And the deposition alone was 21 quite long. I remember like it was yesterday. 22 And, then, Brower was probably about 40 23 hours. 24 Q Okay.</p>
<p style="text-align: right;">Page 23</p> <p>1 Right. 2 Q So the supplemental material list that 3 you brought with you today, Dr. Birrer, is 4 attached to the back of this notice with 5 objections; correct? 6 A That's the same as this. Yes. 7 Q Yes. 8 A Yeah. Uh-huh. 9 Q And also attached to this -- this 10 notice with objections are your fees; correct? 11 A Correct. 12 Q And are -- are those all the invoices 13 that you have submitted thus far? 14 A Yes. 15 Q And how much -- and from -- this 16 invoice that's attached to Exhibit 3 goes through 17 March 17th. 18 How much time would you say you have 19 spent since March 17th preparing for the case? 20 A I'd say probably put another 15 hours, 21 And I haven't invoiced that yet. 22 Q Okay. And you have testified in other 23 cases for the defendants in -- 24 A Correct.</p>	<p style="text-align: right;">Page 25</p> <p>1 A And those invoices are being 2 constructed. 3 Q And you're charging those at the same 4 rate as in your fee schedule -- 5 A That's right. 6 Q -- attached to this document? 7 A That's right. 8 Q Okay. When were you first approached 9 by Johnson & Johnson as -- about serving as an 10 expert in talcum powder litigation? 11 A So that was before the -- that was the 12 Blaes or Swan case. I believe it was in 13 December, around November, December of 2016. 14 Q '16? 15 A Thank you. Time flies. 16 Q Only because I know that the report was 17 submitted in May, so -- 18 A (Nods affirmatively.) 19 Q -- I'm assuming that you didn't work 18 20 months on that -- 21 A No. 22 Q -- case. 23 And you were asked in -- for this 24 report that you just submitted, to address the</p>

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<p style="text-align: right;">Page 26</p> <p>1 biological plausibility of the plaintiffs' theory 2 that cosmetic talcum powder can cause ovarian 3 cancer. Right? 4 A Correct. 5 Q And that would be the stand- -- from 6 the standpoint of the genomics and molecular 7 biology that is your expertise; correct? 8 MS. CURRY: 9 Object to the form. 10 A So I think they were asking me in the 11 big picture the biologic plausibility of talc 12 being involved in the -- causing ovarian cancer 13 and then my scientific experience, even clinical 14 experience, would factor into -- to -- to that 15 expert opinion. 16 MS. THOMPSON: 17 Q Was that a different opinion than what 18 you were asked to provide in the previous cases 19 that you testified in? 20 MS. CURRY: 21 Object to the form. 22 A Previously -- the answer, I believe, is 23 no. But I was asked for general causation 24 before. This was a more -- somewhat more narrow</p>	<p style="text-align: right;">Page 28</p> <p>1 with an increased risk of epithelial ovarian 2 cancer? 3 A Correct. 4 Q Is it your opinion that the genital use 5 of talcum powder is not a risk factor for 6 epithelial ovarian cancer? 7 A Correct. 8 Q Is it your opinion that genital use of 9 talcum powder products does not cause ovarian 10 cancer? 11 A Correct. 12 Q Is it your opinion that the genital use 13 of talcum powder products does not cause ovarian 14 cancer in some women? 15 MS. CURRY: 16 Object to the form. 17 A Correct. 18 MS. THOMPSON: 19 Q And that would be ever. 20 MS. CURRY: 21 Object -- object to the form. 22 A No data to support that. 23 MS. THOMPSON: 24 Q Is it your opinion that the genital use</p>
<p style="text-align: right;">Page 27</p> <p>1 expert opinion. 2 MS. THOMPSON: 3 Q So in this case, you're not providing 4 general causation opinions. You're providing the 5 biological mechanism, plausibility opinions; 6 correct? 7 A Well, the title -- 8 MS. CURRY: 9 Object to the form. 10 A The title on the expert report is for 11 General Causation For the Daubert Hearing. But 12 my understanding was -- was to focus extensively, 13 if you will, on the biologic plausibility. 14 MS. THOMPSON: 15 Q And because biological plausibility is 16 part of general causation; correct? 17 A Correct. 18 Q But it's not the whole of general 19 causation. Is that your understanding? 20 A Correct. 21 Q So I want to make sure that I 22 understand your opinions. 23 Is it your opinion that the perineal 24 use of talcum powder products is not associated</p>	<p style="text-align: right;">Page 29</p> <p>1 of talcum powder does not contribute to the 2 development of epithelial ovarian cancer? 3 A Yes. 4 Q And do you say that there's no data to 5 support that as well? 6 A Correct. 7 Q Is it your opinion that genital use of 8 talcum powder does not contribute to the 9 development of ovarian cancer in some women? 10 MS. CURRY: 11 Object to the form. 12 A There's no data to support that either. 13 MS. THOMPSON: 14 Q So the answer is yes? 15 A Yes. 16 Q Is it your opinion that any proposed 17 biologic mechanism for how the genital use of 18 talcum powder products could cause epithelial 19 ovarian cancer is not plausible? 20 MS. CURRY: 21 Object to the form. 22 A I would agree with that statement. 23 It's not biologically plausible. 24 MS. THOMPSON:</p>

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<p style="text-align: right;">Page 30</p> <p>1 Q Is it your opinion that any proposed</p> <p>2 biologic mechanism for how the genital use of</p> <p>3 talcum powder products might contribute to the</p> <p>4 development of ovarian cancer is not plausible?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A There's no data for that either.</p> <p>8 MS. THOMPSON:</p> <p>9 Q So the answer would be yes?</p> <p>10 A Yes.</p> <p>11 Q Do you intend to give opinions on</p> <p>12 whether talc particles can reach the ovaries?</p> <p>13 A I believe on my expert report and in --</p> <p>14 and I'm more than happy to talk about it --</p> <p>15 reviews the migration theories.</p> <p>16 Q Do you consider yourself to be an</p> <p>17 expert in that area?</p> <p>18 A I think that those studies are</p> <p>19 relatively straightforward and, based upon my</p> <p>20 experience that, I would be relatively easy to</p> <p>21 interpret those.</p> <p>22 Q Do you feel like you would be in a</p> <p>23 better position than a gynecologist or</p> <p>24 gynecologic oncologist?</p>	<p style="text-align: right;">Page 32</p> <p>1 Object to the form.</p> <p>2 A Correct.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Are all the opinions contained in your</p> <p>5 report that you will be providing in this case?</p> <p>6 A That's a tough question to ask because</p> <p>7 I don't know what you're gonna ask me.</p> <p>8 Q Fair enough.</p> <p>9 Can you think of any areas, sitting</p> <p>10 here today, that you intend to testify in other</p> <p>11 than the migration and transport of particles and</p> <p>12 the molecular and genomics of cellular tissue</p> <p>13 response to talc?</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 A Well, that's the bulk of my expert</p> <p>17 report. I'm -- again, it depends on what you ask</p> <p>18 me within the construct of general causation.</p> <p>19 I'm willing to talk about some of that.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Okay. I understand.</p> <p>22 A Uh-huh.</p> <p>23 Q And you are not an epidemiologist;</p> <p>24 correct?</p>
<p style="text-align: right;">Page 31</p> <p>1 A Yes.</p> <p>2 Q Have you found any new expertise in the</p> <p>3 migration or transport of particles in the female</p> <p>4 reproductive system since 2017?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A I'm not sure what you mean by "found</p> <p>8 any new expertise." In the literature or my own</p> <p>9 experience?</p> <p>10 MS. THOMPSON:</p> <p>11 Q Do you believe that you have more</p> <p>12 expertise in that subject than you did in 2017?</p> <p>13 A I think that it's comparable.</p> <p>14 Q So that would be no additional</p> <p>15 expertise since 2017, when you testified</p> <p>16 previously?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A Not that I can identify as -- as we're</p> <p>20 discussing this.</p> <p>21 MS. THOMPSON:</p> <p>22 Q And same for 2018, when you gave a</p> <p>23 deposition in -- in a talcum powder case?</p> <p>24 MS. CURRY:</p>	<p style="text-align: right;">Page 33</p> <p>1 A I don't have a degree in epidemiology.</p> <p>2 But I have training.</p> <p>3 Q So would you agree that your</p> <p>4 understanding of epidemiology is general in</p> <p>5 nature?</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A So in order to be a, you know,</p> <p>9 laboratory-based scientist in this field and a</p> <p>10 clinician to treat patients, you certainly need</p> <p>11 to have an understanding of epidemiologic</p> <p>12 studies, so I have that understanding. And I</p> <p>13 think that it gives me the ability to assess</p> <p>14 epidemiologic studies and to draw conclusions</p> <p>15 from them.</p> <p>16 MS. THOMPSON:</p> <p>17 Q But if you're looking for more nuanced</p> <p>18 or more comprehensive epidemiological experience,</p> <p>19 you would look to an actual epidemiologist;</p> <p>20 correct?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A Well, I think it would depend on the</p> <p>24 question that's being asked.</p>

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<p style="text-align: right;">Page 34</p> <p>1 MS. THOMPSON: 2 Q Well, for example, in the consortium 3 that you publish with, there are specific 4 epidemiologists that publish with the group; 5 correct? 6 A Which consortium are you referring to? 7 Q There are several? 8 A Yes. 9 Q Take -- take the Ovarian Cancer 10 Association Consortium. 11 A The GOS? 12 Q No. OCAC or -- 13 A Okay. 14 Q There are specific epidemiologists that 15 I assume are recruited to -- to provide the 16 epidemiology experience in that consortium; 17 correct? 18 A There are epidemiologists in that 19 consortium. I will point out there are lots of 20 other people and scientists. 21 Q And -- and -- and you would be sought 22 out for that type of consortium because of your 23 molecular experience; correct? 24 MS. CURRY:</p>	<p style="text-align: right;">Page 36</p> <p>1 comments, and they're all listed in terms of 2 biologic plausibility. And then, of course, I 3 spent a lot of time on Dr. Saed. 4 MS. THOMPSON: 5 Q My question, though, is which of the 6 plaintiff experts were you asked to offer 7 criticism of? 8 MS. CURRY: 9 Object to the form. 10 A So I reviewed the entire list, and 11 that's listed in the materials. I think it's on 12 page -- 13 MS. THOMPSON: 14 Q 28? 15 A -- 28 and 29. 16 Q Okay. Let's go ahead and go -- do -- 17 did you read all of these experts -- expert 18 reports? 19 A I looked through them, yes. 20 Q And each one? 21 A Correct. 22 Q All right. Let's go through each one 23 and have you tell me what you gleaned from each 24 expert report.</p>
<p style="text-align: right;">Page 35</p> <p>1 Object to the form. 2 A Well, I would add to that that I think 3 from a -- sort of a clinical standpoint we 4 provide some reality testing in terms of 5 whether -- what they're observing is actually 6 meaningful. 7 MS. THOMPSON: 8 Q Yes. So it would be for your 9 experience as a clinician in genomics and 10 molecular researcher; right? 11 A Yes. 12 Q That makes sense. 13 You're not a gynecologist or 14 gynecologic oncologist; correct? 15 A Correct. 16 Q Were you asked to offer criticism of 17 plaintiff experts and their opinions? 18 MS. CURRY: 19 Object to the form. 20 A So in my expert report, I really 21 reviewed the primary literature, and with -- with 22 then integrating that into the arguments made by 23 plaintiffs' expert witnesses. So you see in a 24 section there I began to look at individuals'</p>	<p style="text-align: right;">Page 37</p> <p>1 MS. CURRY: 2 Object to the form. 3 MS. THOMPSON: 4 Q Ann McTiernan, do you know Ann 5 McTiernan? 6 A I don't know her personally. 7 Q What's her field of expertise? 8 A I would have to check that. 9 Q So you don't remember here today 10 what -- 11 A Well, you're reviewing, I think -- 12 let's be honest, 300 pages. I'm not going to be 13 able to go through those systematically. 14 Q Well -- 15 A But if you look at my report, it very 16 specifically addressed some of the flaws in the 17 experts' opinions regarding migration of talc. 18 Q I -- I understand. But my question is 19 do you know what Dr. McTiernan's area of 20 expertise is? And it's fine if you don't. 21 A I'd have to look it up. 22 Q Okay. Do you know Dr. Carson's area of 23 expertise? 24 A I have never met him, and I don't know</p>

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<p style="text-align: right;">Page 38</p> <p>1 him.</p> <p>2 Q Have you met Dr. McTiernan?</p> <p>3 A No.</p> <p>4 Q What is Dr. Clarke-Pearson's area of</p> <p>5 expertise?</p> <p>6 A Clarke-Pearson is a gynecological</p> <p>7 oncologist, former department chair at UNC. Now</p> <p>8 he's stepped down.</p> <p>9 Q And do you know Dr. Clarke-Pearson?</p> <p>10 A I've met him.</p> <p>11 Q And what about Dr. Kessler?</p> <p>12 A I've never met Dr. Kessler.</p> <p>13 Q What's his area of expertise?</p> <p>14 A I can't quote you that.</p> <p>15 Q What's Dr. Smith's area of expertise?</p> <p>16 A I think Dr. Smith's pretty -- actually,</p> <p>17 I can't tell you.</p> <p>18 Q And Dr. Saed, I think we know.</p> <p>19 What about Dr. Siemiatycki?</p> <p>20 A Uh-uh. No.</p> <p>21 Q Dr. Wolf?</p> <p>22 A I've met Judith. She's a gynecologic</p> <p>23 oncologist.</p> <p>24 Q And do you know Dr. Zelikoff's area of</p>	<p style="text-align: right;">Page 40</p> <p>1 experiments?</p> <p>2 A No. Laboratory-based?</p> <p>3 Q Laboratory, yes.</p> <p>4 A No.</p> <p>5 Q What did you know about talcum powder</p> <p>6 and a possible link to ovarian cancer before you</p> <p>7 were approached to serve as an expert in 2017?</p> <p>8 A So it was not something that we dealt</p> <p>9 with clinically. We never counseled patients.</p> <p>10 Scientifically, it never really was part of my</p> <p>11 laboratory effort. I didn't know really -- I</p> <p>12 didn't know anybody working with it in the lab.</p> <p>13 And -- and, you know, to be fair, I would say</p> <p>14 that I was aware of the sort of concept that some</p> <p>15 people -- some epidemiologic studies were being</p> <p>16 done trying to determine relationship of talc</p> <p>17 exposure to ovarian cancer. And that's about it.</p> <p>18 Q Were you -- were you aware of the</p> <p>19 issues raised by Dr. Woodruff and others in the</p> <p>20 '70s about possible contamination with asbestos?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A No.</p> <p>24 MS. THOMPSON:</p>
<p style="text-align: right;">Page 39</p> <p>1 expertise?</p> <p>2 A I don't know her.</p> <p>3 Q Nor her area of expertise?</p> <p>4 A Correct.</p> <p>5 Q What about Dr. Plunkett? Do you know</p> <p>6 her area of expertise?</p> <p>7 A I don't.</p> <p>8 Q Dr. Moorman, do you know her area of</p> <p>9 expertise?</p> <p>10 A Don't know her. No.</p> <p>11 Q Dr. Smith-Bindman, do you know her area</p> <p>12 of expertise?</p> <p>13 A No.</p> <p>14 Q Do you know the area of expertise of</p> <p>15 Dr. Kane?</p> <p>16 A Nope.</p> <p>17 Q Dr. Levy?</p> <p>18 A No.</p> <p>19 Q Dr. Singh?</p> <p>20 A No.</p> <p>21 Q Were you asked by Johnson & Johnson to</p> <p>22 perform any experiments?</p> <p>23 A No.</p> <p>24 Q Did you offer to perform any</p>	<p style="text-align: right;">Page 41</p> <p>1 Q Did you have any opinions about whether</p> <p>2 talcum powder could cause ovarian cancer before</p> <p>3 you were approached to serve as an expert?</p> <p>4 A Well, my sense was that it wasn't a</p> <p>5 factor.</p> <p>6 Q And what was --</p> <p>7 A Because we -- again, we weren't -- we</p> <p>8 weren't using it in the clinic. We weren't</p> <p>9 talking about it. There were essentially no</p> <p>10 presentations in the biologic plausibility within</p> <p>11 any of the scientific meetings that I would go</p> <p>12 to.</p> <p>13 Q And at that time, that's what your</p> <p>14 impression, at least, would have been based on?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A Yeah.</p> <p>18 MS. THOMPSON:</p> <p>19 Q Did you write your report?</p> <p>20 A Yes.</p> <p>21 Q Every word?</p> <p>22 A Yes.</p> <p>23 Q Did you choose the literature to cite?</p> <p>24 A So I pulled out most of that myself,</p>

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<p style="text-align: right;">Page 42</p> <p>1 went back and did a reference list and then 2 pulled more. As I said before, the expert 3 reports would have been provided from counsel. 4 There may have been some papers that I 5 said, hey, I don't have this. Can you pull this 6 out? And then they would -- they would provide 7 it to me. 8 Q And there are -- just so I understand 9 the literature -- 10 A Uh-huh. 11 Q -- there's literature that you actually 12 cite in the report in footnotes; right? 13 A Correct. 14 Q And then there's another list at the 15 end of the report that's considered -- that's 16 titled "Materials Reviewed and Considered by Dr. 17 Birrer"; right? 18 A That's right. 19 Q And can I assume that the literature 20 that are actually cited in the footnotes is 21 literature that you felt was particularly 22 significant? 23 MS. CURRY: 24 Object to the form.</p>	<p style="text-align: right;">Page 44</p> <p>1 of information, I did that by searching. 2 MS. THOMPSON: 3 Q And what search engines did you use? 4 A It was mostly PubMed, which is 5 something we use all the time. 6 Q And did you -- what search terms did 7 you use? 8 A Ovary, ovarian cancer, talc. So the 9 ones you -- you'd predict. And that doesn't 10 necessarily generate the entire list. Right? I 11 mean, you get the list and then you look at the 12 papers, go back to the references in those 13 papers, and then you see if you -- you're missing 14 out. Then you pull out more. And as you go 15 through this iteration, you begin to find out 16 that you're identifying the same patient -- the 17 same papers. So then you begin to get an idea 18 that you have the sum total of what you need. 19 Q And have you saved those papers 20 anywhere? 21 A So those were -- the way that worked 22 was they came in, mostly computer-based, and then 23 I would look at those, extract what I wanted, and 24 then construct the report. And that was all done</p>
<p style="text-align: right;">Page 43</p> <p>1 A Yeah. So the idea here was to try to 2 provide some guidance as to where that reference 3 was relevant within the document. That's why 4 it's on each page. At the end is a sort of sum 5 total. 6 MS. THOMPSON: 7 Q Okay. 8 A Yeah. 9 Q Did you choose any quotes that are 10 included in your expert report yourself? 11 MS. CURRY: 12 Object to the form. 13 MS. THOMPSON: 14 Q It was a bad question. 15 Did you choose the quotes that are 16 included in your expert report? 17 A Correct. 18 Q Did you choose the language that you 19 used to criticize the plaintiffs' experts? 20 A Correct. 21 Q Did you perform any searches? 22 MS. CURRY: 23 Object to the form. 24 A In order to generate the original body</p>	<p style="text-align: right;">Page 45</p> <p>1 in the computer. 2 Q But what happened to the articles? 3 MS. CURRY: 4 Object to the form. 5 A Well, they'd be computer-based, or 6 there's backup, I believe, some backup copies 7 here on everything. 8 MS. THOMPSON: 9 Q So -- so everything that you looked at 10 would be in your materials considered list and 11 the supplemental materials considered list? 12 A Correct. Yep. 13 Q Did you look at plaintiff expert 14 depositions? 15 A Correct. 16 Q Which ones? 17 A So I looked at the deposition of 18 Dr. Saenz. I think that's listed on supplemental 19 deposition. 20 MS. CURRY: 21 I believe she asked about plaintiff 22 expert deposition. 23 MS. THOMPSON: 24 Q Plaintiff.</p>

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<p>1 A I'm sorry. I'm on the wrong one. So</p> <p>2 that would be Dr. Saed.</p> <p>3 Q Uh-huh.</p> <p>4 A And I think -- let's go back and look.</p> <p>5 I think -- yeah. It was 23 and 24 are -- were</p> <p>6 both the Saed depositions. I think that's it.</p> <p>7 Q In the file -- the backup file that you</p> <p>8 mentioned that's here, is that on a thumb drive</p> <p>9 or what's --</p> <p>10 MS. CURRY:</p> <p>11 Object to the form. They're actually</p> <p>12 my -- the lawyer's files. I just brought a copy</p> <p>13 of the references in case we needed to refer to</p> <p>14 everything. But it's not -- actually not</p> <p>15 Dr. Birrer's file.</p> <p>16 MS. THOMPSON:</p> <p>17 Q So there's no electronic file that you</p> <p>18 possess?</p> <p>19 A Yeah.</p> <p>20 Q Did you make any notes or highlights on</p> <p>21 any of the articles that --</p> <p>22 A (Shakes head negatively.)</p> <p>23 Q And in addition to Dr. Saed's</p> <p>24 deposition, you have listed two drafts of his</p>	<p>1 MS. CURRY:</p> <p>2 Here you go.</p> <p>3 A This supplemental list with objections</p> <p>4 or the extra paper?</p> <p>5 MS. THOMPSON:</p> <p>6 Q And you reviewed some reports from</p> <p>7 governmental and regulatory agencies; correct?</p> <p>8 A Correct.</p> <p>9 Q I'll go ahead and mark those. We're</p> <p>10 gonna discuss them more later.</p> <p>11 (DEPOSITION EXHIBIT NUMBER 4</p> <p>12 WAS MARKED FOR IDENTIFICATION.)</p> <p>13 MS. THOMPSON:</p> <p>14 Q You've looked at the Health Canada's</p> <p>15 recent draft assessment; correct?</p> <p>16 A Yes.</p> <p>17 Q When did you first see that?</p> <p>18 A It was in a deposition of Dr. Saenz's.</p> <p>19 Q And do you know when that was first</p> <p>20 published?</p> <p>21 A The Health Canada?</p> <p>22 Q Yes.</p> <p>23 A Fairly recently. Can't quote you the</p> <p>24 date.</p>
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<p>1 manuscript that was recently published; correct?</p> <p>2 A I believe I saw the pre-print and then</p> <p>3 the copy of the actual published paper. And, of</p> <p>4 course, his expert report.</p> <p>5 Q When did you first see Dr. Saed's</p> <p>6 manuscript?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Preprint or published?</p> <p>10 MS. THOMPSON:</p> <p>11 Q Either.</p> <p>12 A So I think the preprint came first,</p> <p>13 obviously. The expert report was available</p> <p>14 first, and then the preprint, and then just</p> <p>15 within, I think, a month and a half I got the</p> <p>16 paper. It was pretty recent.</p> <p>17 Q Is Dr. Saenz's published manuscript on</p> <p>18 your supplemental materials list?</p> <p>19 MS. CURRY:</p> <p>20 It's attached to the objections, which</p> <p>21 is Exhibit 3.</p> <p>22 MS. THOMPSON:</p> <p>23 Yeah. I -- I couldn't find my notice</p> <p>24 with objections.</p>	<p>1 Q If it was December, would that surprise</p> <p>2 you?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A December of --</p> <p>6 MS. THOMPSON:</p> <p>7 Q Of '18?</p> <p>8 A That's pretty recent.</p> <p>9 Q Were you not aware that this had been</p> <p>10 put online by Health Canada prior to Dr. Saenz's</p> <p>11 deposition?</p> <p>12 A I was not.</p> <p>13 Q Did you review that 2014 letter from</p> <p>14 FDA in response to a public citizen complaint?</p> <p>15 A I am familiar with that.</p> <p>16 (DEPOSITION EXHIBIT NUMBER 5</p> <p>17 WAS MARKED FOR IDENTIFICATION.)</p> <p>18 MS. THOMPSON:</p> <p>19 Q And I'll mark that 2014 public citizen</p> <p>20 response letter from the FDA as Exhibit Number 5.</p> <p>21 Does that look like the letter that you</p> <p>22 reviewed, Dr. Birrer?</p> <p>23 A (Nods affirmatively.) I've seen that,</p> <p>24 yeah.</p>

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<p style="text-align: right;">Page 50</p> <p>1 Q And did you review the IARC Monograph 2 on Nonasbestiform Talc from 2010? 3 A I did. 4 Q And that will be Exhibit Number 6. 5 (DEPOSITION EXHIBIT NUMBER 6 6 WAS MARKED IDENTIFICATION.) 7 MS. THOMPSON: 8 Q Does that look like the document that 9 you reviewed? 10 A Yes. Yeah. I've seen that. Yep. 11 MS. THOMPSON: 12 Dawn, if you want more copies, I'm 13 happy to give -- 14 MS. CURRY: 15 I'm okay. I don't know if other 16 counsel need a copy to review. 17 MR. MIZGALA: 18 No. 19 MS. THOMPSON: 20 I think for most everything I have 21 another copy, so if there's anything you'd like 22 to see and not have to take home with you, I'm 23 happy to provide it. 24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 52</p> <p>1 Q Okay. That's my question. 2 A Yes. 3 Q But it was published in December, and 4 you didn't look at it until you saw it in 5 Dr. Saenz's deposition as an exhibit; right? 6 A Correct. 7 Q Did you deem it important? 8 MS. CURRY: 9 Object to the form. 10 A Well, since it was quoted and my 11 impression was that there were people who thought 12 this was important, that necessitated me to take 13 a look at it. 14 MS. THOMPSON: 15 Q Did you think it was important? 16 MS. CURRY: 17 Object to the form. 18 A Well, after I read it, again, my sense 19 was it doesn't really sway me one more -- one way 20 or the other because they're -- they're 21 essentially re-reviewing all the data that we 22 know and coming to a different conclusion. I 23 just think they got it wrong, unfortunately. 24 MS. THOMPSON:</p>
<p style="text-align: right;">Page 51</p> <p>1 Q Did you know that the Health Canada 2 assessment was made pub- -- made available to the 3 public? 4 A Yes. 5 MS. CURRY: 6 Object to the form. 7 MS. THOMPSON: 8 Q Do you believe that the Health Canada 9 risk assessment is relevant to the topic today? 10 MS. CURRY: 11 Object to the form. 12 A It doesn't change my opinion about 13 biologic plausibility. It's a -- obviously, an 14 opinion that's based upon a lot of data that I 15 believe is reviewed by Taher, which is 16 information data that I already was aware of, so 17 it doesn't really sway me one way or the other. 18 MS. THOMPSON: 19 Q But my question was, did you deem it 20 relevant? 21 MS. CURRY: 22 Object to the form. 23 A Relevant to review. 24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 53</p> <p>1 Q But you will agree that it did provide 2 an extensive review on the subject? 3 MS. CURRY: 4 Object to the form. 5 A It was, I thought, would be described 6 as extensive. 7 MS. THOMPSON: 8 Q Did you review the statement of the 9 methodology that accompanied the risk assessment? 10 A I went -- I looked through it. 11 Q I'll mark that as Exhibit 7. 12 (DEPOSITION EXHIBIT NUMBER 7 13 WAS MARKED IDENTIFICATION.) 14 MS. THOMPSON: 15 Q Is that what you saw? 16 A I didn't see it printed like this with 17 the color on it. Yeah. 18 Q And let's just look at page 2 of the 19 document titled "Weight of Evidence, General 20 Principles and Current Applications in Health 21 Canada." 22 Does number 3, Role in Risk 23 Assessments, generally outline the methodology 24 that Health Canada applied to this risk</p>

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<p style="text-align: right;">Page 54</p> <p>1 assessment?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 A Yeah. I think it's a summary of</p> <p>5 what -- of how they approached it. That's my</p> <p>6 sense. Yep.</p> <p>7 MS. THOMPSON:</p> <p>8 Q And for the risk assessment, Health</p> <p>9 Canada assumed talc or talcum products to be</p> <p>10 nonasbestiform.</p> <p>11 Is that your understanding?</p> <p>12 A Yeah. I believe that's what they</p> <p>13 focused on.</p> <p>14 Q What does nonasbestiform mean?</p> <p>15 A I'm not going to go down the line of</p> <p>16 being an expert in asbestos.</p> <p>17 Q So do you not know what it means when</p> <p>18 the talc is considered nonasbestiform?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A I'm assuming they're addressing sort of</p> <p>22 mineral characterization of these substances.</p> <p>23 But again, I -- that's not my area of expertise.</p> <p>24 I'm not a geologist and it -- it in many ways is</p>	<p style="text-align: right;">Page 56</p> <p>1 MS. THOMPSON:</p> <p>2 Q So you're agreeing it's irrelevant what</p> <p>3 form the particles are in when --</p> <p>4 A I'm saying we don't have any data.</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 You have to let her get her --</p> <p>8 THE WITNESS:</p> <p>9 Okay.</p> <p>10 MS. CURRY:</p> <p>11 -- entire question out before you</p> <p>12 answer so that the court reporter can get</p> <p>13 everything down.</p> <p>14 MS. THOMPSON:</p> <p>15 Q No data isn't the same as irrelevant,</p> <p>16 and that's my question.</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A You know, again, I don't think I can</p> <p>20 answer that "yes" or "no."</p> <p>21 MS. THOMPSON:</p> <p>22 Q Is it important whether the substance</p> <p>23 in Johnson's baby powder and Shower to Shower is</p> <p>24 in a particulate form or in a fiber form?</p>
<p style="text-align: right;">Page 55</p> <p>1 sort of irrelevant to looking at many of the</p> <p>2 studies which are just looking at talcum powder.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Does it not matter to you whether that</p> <p>5 talc is in a particle or fiber -- fiber form?</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A Well, I looked at, again, extensively</p> <p>9 all the data that was addressing whether talcum</p> <p>10 powder is a risk factor or plays a role in</p> <p>11 developing ovarian cancer. It is irrelevant in</p> <p>12 that setting whether there are components in</p> <p>13 there that go from asbestiform to heavy metals to</p> <p>14 fragrance. That data would be clear from those</p> <p>15 experiments, and they're not.</p> <p>16 MS. THOMPSON:</p> <p>17 Q So is the answer that -- is it</p> <p>18 irrelevant whether the particles are in a</p> <p>19 particulate form or in a fiber form?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A Again, I -- that -- that experiment has</p> <p>23 not been done in the -- the -- in the -- in the</p> <p>24 data that I looked at.</p>	<p style="text-align: right;">Page 57</p> <p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A I don't know.</p> <p>4 MS. THOMPSON:</p> <p>5 Q You don't know if it's important?</p> <p>6 A I don't know if it's important.</p> <p>7 Q Okay. And is part of the reason is</p> <p>8 because you're not an expert in asbestos?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A Again, I wasn't asked to evaluate the</p> <p>12 role of asbestos in ovarian cancer. I have an</p> <p>13 opinion on that based upon some of the</p> <p>14 epidemiologic studies.</p> <p>15 But in terms of the compositional</p> <p>16 analysis of talcum powder, that is not within the</p> <p>17 area of my expertise, and the various forms of</p> <p>18 asbestos in talc in terms of mineralogy is not</p> <p>19 something that I've spent time on.</p> <p>20 But, as I pointed out before, the</p> <p>21 experiments that have been conducted address that</p> <p>22 issue, which is they're using talcum powder. If</p> <p>23 it's got a variety of substances in it, any one</p> <p>24 of which match and play a role in ovarian cancer,</p>

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<p style="text-align: right;">Page 58</p> <p>1 it would have been obvious from the data and it's</p> <p>2 not.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Is it your opinion that baby powder and</p> <p>5 Shower to Shower -- and you understand those are</p> <p>6 the two products that we're here to talk about</p> <p>7 today; right?</p> <p>8 A Yes. J & J products?</p> <p>9 Q Yes.</p> <p>10 Is it your opinion that those products</p> <p>11 have been proven safe?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A So there's no data that I know of that</p> <p>15 says they're not safe.</p> <p>16 MS. THOMPSON:</p> <p>17 Q That's different. Have they been</p> <p>18 proven safe?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A Yes.</p> <p>22 MS. THOMPSON:</p> <p>23 Q And what data do you have as the basis</p> <p>24 for that, that they have been proven safe?</p>	<p style="text-align: right;">Page 60</p> <p>1 has it been proven unsafe, so --</p> <p>2 MR. MIZGALA:</p> <p>3 Object to the form.</p> <p>4 MS. THOMPSON:</p> <p>5 Q -- I'll ask the question again.</p> <p>6 Have these products been proven safe in</p> <p>7 your mind?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Again, it is -- it is an issue about</p> <p>11 trying to prove a negative. The data is there</p> <p>12 are decades of use of this, this material,</p> <p>13 perineal dusting, with no evidence, no convincing</p> <p>14 evidence that it's unsafe. I conclude that it's</p> <p>15 a safe product.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Do you believe that the molecular data</p> <p>18 proves the product safe?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A Can you define "molecular data"?</p> <p>22 MS. THOMPSON:</p> <p>23 Q The -- the studies that have been</p> <p>24 performed on talcum powder, do you believe they</p>
<p style="text-align: right;">Page 59</p> <p>1 A Again, years and years of usage with</p> <p>2 these experiments and biologic systems,</p> <p>3 epidemiologic data is basically not exposing or</p> <p>4 uncovering any definitive data that that they're</p> <p>5 unsafe.</p> <p>6 Q So you believe the epidemiological data</p> <p>7 proves the product safe?</p> <p>8 A I don't think it -- it proves that it's</p> <p>9 a risk factor.</p> <p>10 Q Is that --</p> <p>11 A You're asking -- you're asking me to</p> <p>12 prove a negative. I can't do that.</p> <p>13 Q So you're not -- you're unable to prove</p> <p>14 that it's safe because you can't prove a</p> <p>15 negative?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 MS. THOMPSON:</p> <p>19 Q Is that what you're saying?</p> <p>20 A I get -- yeah. I think -- I think the</p> <p>21 issue in front of us is: Is it unsafe? And the</p> <p>22 answer to that is there's no data for it.</p> <p>23 Q Well, the issue is what I asked you.</p> <p>24 And my question was has it been proven safe, not</p>	<p style="text-align: right;">Page 61</p> <p>1 prove that the products are safe?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 A Just repeat that once more, please.</p> <p>5 MS. THOMPSON:</p> <p>6 Q The molecular studies that have been</p> <p>7 done on talcum powder, is it your opinion that</p> <p>8 they prove that the products are safe?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A So I refine that a bit because I don't</p> <p>12 really consider them molecular studies. They're</p> <p>13 biologic studies, and there's a difference.</p> <p>14 The biologic studies which I reviewed,</p> <p>15 which I think is the sum total that's out there,</p> <p>16 are completely unconvincing, unconvincing that</p> <p>17 talcum powder is a -- plays a role in the</p> <p>18 development of ovarian cancer.</p> <p>19 MS. THOMPSON:</p> <p>20 Q But my question was is it your belief</p> <p>21 that the biologic studies confirm that the</p> <p>22 product is safe?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>

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<p style="text-align: right;">Page 62</p> <p>1 A Again, we're back sort of to that 2 negative. I -- I think if -- I don't think they 3 convince me at all that it's -- it's a risk or 4 that it has any biologic activity on the target 5 organ, which is the ovary. And then in the 6 context of decades of use, then I would conclude 7 that it's a safe product. 8 MS. THOMPSON: 9 Q And it's fine to say you can't 10 answer -- you can't answer the question. But I 11 need -- but I want to have an answer. 12 And that is: Is it your opinion that 13 the biologic studies show that the products are 14 safe? 15 MS. CURRY: 16 Object to the form. 17 A Yeah. I -- I think -- I think 18 certainly that -- I think we can say that the 19 biologic studies do not reveal any untoward 20 effects. It's not reliable. The experiments are 21 not reliable. And so in that context, it's a 22 safe product. 23 I mean, again, you're asking me for a 24 biologic experiment that proves something is</p>	<p style="text-align: right;">Page 64</p> <p>1 reviewing the assessment? 2 A I believe so, but let me just -- 3 MS. CURRY: 4 Do you have the marked Exhibit 4 there? 5 I don't think the witness actually has 6 the -- 7 Oh, I think it's in front of you here. 8 I'm just gonna grab these marked 9 exhibits for him. Thank you. 10 MS. THOMPSON: 11 I think his is the marked exhibit, 12 unless I -- 13 MS. CURRY: 14 Right. It was just in front of you. 15 MS. THOMPSON: 16 Oh, I -- yeah. 17 MS. CURRY: 18 He didn't have it. That's all. 19 MS. THOMPSON: 20 Sorry. 21 A Yeah, this -- okay. 22 Yeah. So they -- they essentially went 23 through it in that kind of algorithm. 24 MS. THOMPSON:</p>
<p style="text-align: right;">Page 63</p> <p>1 safe. I don't even know how to conduct an 2 experiment like that. 3 MS. THOMPSON: 4 Q Okay. And again, you know, I can't 5 answer that -- your question -- 6 A It's okay? 7 Q -- is a fine answer. Yeah. 8 MS. CURRY: 9 Object to the form. 10 MS. THOMPSON: 11 Q Back to the weight of the evidence 12 document, it's your understanding that this is 13 the evaluation that Health Canada applied to -- 14 A That's this one? 15 Q Yeah. 16 -- to answering the -- the question of 17 whether talcum powder was a risk for the public 18 in Canada; correct? 19 MS. CURRY: 20 Object to the form. 21 A Correct. 22 MS. THOMPSON: 23 Q And they also applied a Bradford Hill 24 analysis? Is that your understanding from</p>	<p style="text-align: right;">Page 65</p> <p>1 Q I did not see any discussion in your 2 report of a methodology similar to this. Is that 3 right? 4 A Correct. 5 Q Did you perform a weight of the 6 evidence of the data in this case? 7 A So I approached the expert report based 8 upon my experience, both scientifically and 9 clinical. We do this -- we do this a lot, 10 actually, where we'll do a complete review of the 11 literature and then extract the information, 12 dissect it in terms of paper by paper. 13 As a scientist, we don't really weigh 14 studies in a quantitative way. We don't -- it's 15 really not like a meta-analysis where we're 16 saying, okay, this is -- this is this weight 17 versus that weight. 18 But -- but the gestalt is, if you will, 19 at the end of the day, we look at these studies 20 and say do we believe -- do we think that the 21 data and results are believable; do they -- do 22 they support the conclusions. And we do that 23 individually through all the studies. 24 And my expert report, I think, outlines</p>

17 (Pages 62 to 65)

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<p style="text-align: right;">Page 66</p> <p>1 that very clearly.</p> <p>2 So I guess the answer to your question</p> <p>3 is at the end of the day, the conclusion is that</p> <p>4 we don't think -- I don't think the data supports</p> <p>5 a biologic plausibility for talc versus -- talc</p> <p>6 and the -- as a role in the development of</p> <p>7 ovarian cancer. That's the sum total of all that</p> <p>8 analysis.</p> <p>9 Q Did you perform a Bradford Hill</p> <p>10 analysis, per se?</p> <p>11 A Not in the expert report. It's really</p> <p>12 focused on biologic plausibility. I'm aware of</p> <p>13 Bradford Hill. Prior depositions, we talked</p> <p>14 about the elements, and I feel like I -- I</p> <p>15 certainly understand those criteria.</p> <p>16 Q But at least in this report, you didn't</p> <p>17 apply the criteria to this subject?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A It's really focused on biologic</p> <p>21 plausibility, which, as you know, is one</p> <p>22 component of it.</p> <p>23 MS. THOMPSON:</p> <p>24 Q Correct.</p>	<p style="text-align: right;">Page 68</p> <p>1 Q Is it a credible scientific</p> <p>2 organization?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A I -- I think, to be fair, they -- they</p> <p>6 recognize this as a group that is careful and is</p> <p>7 invested in this. I would say, though, that</p> <p>8 they're not, as an organization, completely free</p> <p>9 of -- because of the way they're structured with</p> <p>10 WHO, completely free of outside influence or</p> <p>11 politics. That's my sense.</p> <p>12 MS. THOMPSON:</p> <p>13 Q And by outside influence and politics,</p> <p>14 where would that be coming from?</p> <p>15 A From World Health Organization, which</p> <p>16 is their sort of supervising body.</p> <p>17 Q And is it your belief that the World</p> <p>18 Health Organization is politically biased or</p> <p>19 subject to influence from outside?</p> <p>20 A Well, I think it's an organization</p> <p>21 that, by its nature, is, you know, a compendium</p> <p>22 of countries and societies. And, so, it's --</p> <p>23 let's just say it's not necessarily as sort of</p> <p>24 independent as the Academy, National Academy.</p>
<p style="text-align: right;">Page 67</p> <p>1 And you reviewed that IARC 2010</p> <p>2 document that we've marked as an exhibit; right.</p> <p>3 A This is when it was labeled as 2B;</p> <p>4 right?</p> <p>5 Q Yes.</p> <p>6 And -- and this -- well, this monograph</p> <p>7 was published in 2010; right?</p> <p>8 A Correct.</p> <p>9 Q Is it your understanding that it</p> <p>10 considered literature up to 2006? Correct?</p> <p>11 A Sounds about right, yes.</p> <p>12 Q What is IARC?</p> <p>13 A Well, it's an international agency for</p> <p>14 research on cancer. Part of what they -- their</p> <p>15 responsibility is is to look at environmental</p> <p>16 risks for -- and -- and to sort of attempt to</p> <p>17 quantify them, identify them and quantify them</p> <p>18 for the development of cancer.</p> <p>19 Q Is it generally thought to be a</p> <p>20 reputable scientific organization?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A How do you define "reputable"?</p> <p>24 MS. THOMPSON</p>	<p style="text-align: right;">Page 69</p> <p>1 Q And by that you mean the National</p> <p>2 Academy of Science and Medicine Engineering, now</p> <p>3 titled?</p> <p>4 A Yes.</p> <p>5 Q Okay. And I believe we talked about</p> <p>6 before this --</p> <p>7 A Uh-huh.</p> <p>8 Q -- this monograph applies to talc not</p> <p>9 containing asbestiform fibers, but that is not</p> <p>10 your area of expertise; correct?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A Correct.</p> <p>14 MS. THOMPSON:</p> <p>15 Q And you are aware that there's a</p> <p>16 different IARC monograph published in 2012 that</p> <p>17 would cover talc containing asbestos or talc</p> <p>18 containing asbestiform fibers; correct?</p> <p>19 A I don't think I've seen that.</p> <p>20 Q That would be 2012, the 100C. I</p> <p>21 believe it's on your --</p> <p>22 A Is it?</p> <p>23 Q -- reliance list.</p> <p>24 A Do you have a copy?</p>

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<p>1 Q Yeah. It's number 77.</p> <p>2 A 77.</p> <p>3 Q Arsenic, Metals, Fibers and Dust?</p> <p>4 A Oh, I think I -- I'm sorry. That's</p> <p>5 coming back to me. It was a small -- yeah.</p> <p>6 Q And did you -- did you review that IARC</p> <p>7 monograph?</p> <p>8 A Yeah. There was a -- what -- what</p> <p>9 I looked at was a subset of the entire document.</p> <p>10 Yeah.</p> <p>11 Q Did you look at the section with</p> <p>12 asbestos?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A I believe so, yeah.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Did you look at the section with heavy</p> <p>18 metals?</p> <p>19 A No.</p> <p>20 Q Are you aware that that document, 2012,</p> <p>21 100C, includes all forms of asbestos and talc</p> <p>22 containing asbestiform fibers?</p> <p>23 A That sounds correct.</p> <p>24 Q But you're not sure about that today?</p>	<p>1 Object to the form.</p> <p>2 A It's detailed.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Going to the FDA response letter, at</p> <p>5 least by volume, would you agree that this FDA</p> <p>6 letter is a less extensive review?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Less pages.</p> <p>10 MS. THOMPSON:</p> <p>11 Q That's kind of what I was getting at.</p> <p>12 How about references?</p> <p>13 A Yeah.</p> <p>14 Q So, essentially, the FDA response</p> <p>15 letter in 2014 does not include a description of</p> <p>16 the methodology or an extensive reference list.</p> <p>17 Is that fair --</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 MS. THOMPSON:</p> <p>21 Q -- statement?</p> <p>22 A Well, I -- again, I think a little bit</p> <p>23 you're comparing apples and oranges in the sense</p> <p>24 that the purpose for these documents is somewhat</p>
Page 71	Page 73
<p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A Well, as I said, I'm not a asbestos</p> <p>4 expert. But that -- that IARC volume is focused</p> <p>5 on fibers, so that makes sense.</p> <p>6 MS. THOMPSON:</p> <p>7 Q And have you reviewed the preamble to</p> <p>8 the IARC monographs? It's included in --</p> <p>9 A Yeah.</p> <p>10 Q -- in exhibit --</p> <p>11 A I looked through it.</p> <p>12 Q Okay.</p> <p>13 A It's voluminous.</p> <p>14 Q And does that describe the -- the</p> <p>15 methodology that IARC applies when it's looking</p> <p>16 to determine whether a substance is carcinogenic</p> <p>17 or not?</p> <p>18 A Yes. It's a list of all the</p> <p>19 participants, the general principles, the</p> <p>20 methodology.</p> <p>21 Q And you would agree, similar to Health</p> <p>22 Canada, that that methodology is extensive as</p> <p>23 well?</p> <p>24 MS. CURRY:</p>	<p>1 different in that this is a letter from the FDA</p> <p>2 in response to a -- I think it was a citizen's</p> <p>3 petition. They're not gonna give -- they're not</p> <p>4 gonna send this back to a citizen's petition</p> <p>5 because I think the citizen's petition would be</p> <p>6 insulted because they're not going to be able to</p> <p>7 read it. It's more of a letter than the -- what</p> <p>8 their opinion is.</p> <p>9 Oh. Sorry.</p> <p>10 Q And you're referring to that IARC --</p> <p>11 A Yeah.</p> <p>12 Q -- 2010 monograph. Yeah.</p> <p>13 A Yeah.</p> <p>14 Q Fair enough.</p> <p>15 However, you would consider the FDA a</p> <p>16 credible source?</p> <p>17 A Yes.</p> <p>18 Q Let's look at your CV. And you have</p> <p>19 been a prolific researcher. Would you agree?</p> <p>20 A I survive.</p> <p>21 Q I -- I think there are approximately</p> <p>22 400 published papers. Is that close?</p> <p>23 A Correct.</p> <p>24 Q You have a lot of coauthors on these</p>

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<p>1 papers. Am I right?</p> <p>2 A Correct.</p> <p>3 Q On some, you're the lead author;</p> <p>4 correct?</p> <p>5 A Correct.</p> <p>6 Q What does the role of lead author</p> <p>7 usually entail?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A So let me -- let me step back and</p> <p>11 define that. I would say anchor positions.</p> <p>12 MS. THOMPSON:</p> <p>13 Q Okay.</p> <p>14 A So first author is usually the person</p> <p>15 who has done most of the work. And, it</p> <p>16 actually -- my first authorship positions have</p> <p>17 sort of faded with time because I take the other</p> <p>18 anchor position, which is the senior author,</p> <p>19 where you're providing guidance, mentorship, and</p> <p>20 then you -- you ultimately are responsible for</p> <p>21 the quality of the paper.</p> <p>22 Q And -- and that --</p> <p>23 A Yeah.</p> <p>24 Q -- that person is -- is often listed</p>	<p>1 A No. I think OCAC is a lot like that.</p> <p>2 MS. THOMPSON:</p> <p>3 Q They're providing tissue samples or are</p> <p>4 they providing expertise?</p> <p>5 A Well, OCAC is the consortium, so</p> <p>6 it's -- it's composed of all of those</p> <p>7 institutions. And those institutions are</p> <p>8 providing specimens. And then the authors from</p> <p>9 those institutions end up on the paper.</p> <p>10 Q How are the authors of the consortium's</p> <p>11 publications selected?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A Specific in GWAS or in general?</p> <p>15 MS. THOMPSON:</p> <p>16 Q In OCAC.</p> <p>17 A OCAC. Well, I'm not sure I can quote</p> <p>18 you OCAC rules, but the general guidelines would</p> <p>19 be that from every institution that participated,</p> <p>20 there'd be a primary author. If -- if there was</p> <p>21 somebody else at the institution who specifically</p> <p>22 did something important for that paper, they</p> <p>23 might take two authors. But usually there's a</p> <p>24 limit because you just -- OCAC, I believe, has --</p>
Page 75	Page 77
<p>1 last. Is that right?</p> <p>2 A That's right.</p> <p>3 Q Okay. And can I assume that the</p> <p>4 authors in the middle have varying roles but all</p> <p>5 participate in the preparation of the manuscript</p> <p>6 in some sense?</p> <p>7 A Right. I mean, it becomes -- you</p> <p>8 probably can guess -- somewhat problematic when</p> <p>9 you look at GY studies when there are almost more</p> <p>10 authors than specimens. So the idea there is</p> <p>11 that the individuals in -- in between are still</p> <p>12 contributing to the paper. They're -- they may</p> <p>13 be providing specimens.</p> <p>14 Q And I believe in GWAS, the -- the</p> <p>15 recruitment for GWAS are researchers that can</p> <p>16 provide tissue specimens for the group that's</p> <p>17 analyzing them. Is that a fair --</p> <p>18 A It's a big point. It's -- it's a big</p> <p>19 part of it. Yeah.</p> <p>20 Q And you'd agree that that's different</p> <p>21 from the consortium that we discussed earlier,</p> <p>22 that OCAC consortium; right?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p>1 I'm guessing -- 50 to maybe even 100</p> <p>2 institutions. So if you were to allow unlimited</p> <p>3 authors, it would be unmanageable.</p> <p>4 Q Would the authors typically be</p> <p>5 considered to have expertise in the particular</p> <p>6 area that they're publishing in?</p> <p>7 A Yes.</p> <p>8 Q Would they typically have previous</p> <p>9 scholarly work or publications?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A Usually.</p> <p>13 MS. THOMPSON:</p> <p>14 Q Would they typically have a -- a good</p> <p>15 reputation in the scientific or medical</p> <p>16 community?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A I hope so.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Would they typically be knowledgeable</p> <p>22 in that respective field that they're called upon</p> <p>23 to contribute to the --</p> <p>24 MS. CURRY:</p>

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<p>1 Object to the form.</p> <p>2 A Yeah. I mean, I think it would be</p> <p>3 very -- again, these GWAS studies -- I'm sorry --</p> <p>4 the GWAS studies are in some ways really unique</p> <p>5 in that there's so many authors. There may be</p> <p>6 individuals in that list who -- who while they're</p> <p>7 ovarian cancer researchers, they could be fairly</p> <p>8 junior, and they may have just provided some</p> <p>9 specimens. Yeah.</p> <p>10 MS. THOMPSON:</p> <p>11 Q Yeah. And I'm not as interested in the</p> <p>12 GWAS because they do have, you know, a whole</p> <p>13 number.</p> <p>14 A Yeah.</p> <p>15 Q But I'm thinking more of the Australian</p> <p>16 consortium, the OCAC, the -- the other ones where</p> <p>17 it looks, at least by appearance, that you're --</p> <p>18 the authors are chosen because they're experts</p> <p>19 in -- in a particular area. For example,</p> <p>20 epidemiology. Would you agree with that</p> <p>21 statement?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A I think that's true -- I think that's</p>	<p>1 of careful thought.</p> <p>2 MS. THOMPSON:</p> <p>3 Q And -- and I'd assume they'd be</p> <p>4 qualified in their area of expertise for the same</p> <p>5 reason, or else you wouldn't choose them. Right?</p> <p>6 A It would be hard for them to contribute</p> <p>7 in a meaningful way if they don't know what</p> <p>8 they're doing.</p> <p>9 Q Okay. Looking at your CV, are there</p> <p>10 any coauthors that you can identify that you</p> <p>11 would not regard as qualified in their respective</p> <p>12 fields?</p> <p>13 A I'm not gonna be able to answer that.</p> <p>14 I've got 400 publications and probably several</p> <p>15 thousand authors.</p> <p>16 Q So do you think there would be some</p> <p>17 that you could identify as not being credible?</p> <p>18 A Not that I know of.</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A Again, this is realtime, so if we go</p> <p>22 back to my Ph.D., which was on the measles virus</p> <p>23 back when I was a young lad, I don't know that</p> <p>24 field anymore, and I don't know what those</p>
Page 79	Page 81
<p>1 true as a -- as general guideline, yeah.</p> <p>2 MS. THOMPSON:</p> <p>3 Q And would the same be true for a paper</p> <p>4 that you're publishing? Would you look for</p> <p>5 coauthors -- either as an anchor or a senior,</p> <p>6 would you look for coauthors that are credible?</p> <p>7 A Well, you know, when you do these</p> <p>8 experiments, you're not really out looking for</p> <p>9 authors. You're doing the experiments, and the</p> <p>10 people who do them, help you design a project,</p> <p>11 deserve authorship. Those are the guidelines.</p> <p>12 And if you're asking would I put</p> <p>13 somebody who I thought was not credible on an</p> <p>14 author list, I'd be very bothered by that. But</p> <p>15 you'd have to define what "credible" means.</p> <p>16 Q Yeah. So I guess rather than choosing</p> <p>17 someone as a coauthor, I should have rephrased</p> <p>18 that. Choosing someone to work on a project that</p> <p>19 would later be published, you can assume that</p> <p>20 person would be credible; correct?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A Yeah. I choose my collaborators, like</p> <p>24 others, other scientists, with a certain amount</p>	<p>1 individuals have done.</p> <p>2 It's a realtime process. Sometimes</p> <p>3 individuals who seem to be very, very good</p> <p>4 scientists later on in life will get involved in</p> <p>5 scientific misconduct. That may not have been at</p> <p>6 all relevant for when you put that person on your</p> <p>7 paper.</p> <p>8 (DEPOSITION EXHIBIT NUMBER 8</p> <p>9 WAS MARKED IDENTIFICATION.)</p> <p>10 MS. THOMPSON:</p> <p>11 Q I'm gonna just give you a list of some</p> <p>12 coauthors that I pulled off your CV. And would</p> <p>13 you look at that list?</p> <p>14 A Uh-huh.</p> <p>15 Q I narrowed it down from a couple</p> <p>16 thousand to a more manageable number. Are there</p> <p>17 any names on that list that you could identify as</p> <p>18 not being credible?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 MS. THOMPSON:</p> <p>22 Q And that list is marked as Exhibit --</p> <p>23 Dr. Birrer, can you --</p> <p>24 A 8.</p>

21 (Pages 78 to 81)

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<p style="text-align: right;">Page 82</p> <p>1 Q -- 8.</p> <p>2 A So I would say of this list,</p> <p>3 probably -- I'm estimating -- about 20 percent of</p> <p>4 these people, I'm -- I'm not sure I quite</p> <p>5 remember what paper they're on. But the rest of</p> <p>6 them I know because they're high profile. I</p> <p>7 don't see anybody here that I would say is not a</p> <p>8 good scientist.</p> <p>9 Q And qualified in their respective</p> <p>10 areas?</p> <p>11 A Yes.</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 MS. THOMPSON:</p> <p>15 Q And some -- at least some on the list</p> <p>16 you published with multiple times. Is that fair</p> <p>17 to say?</p> <p>18 A Yeah.</p> <p>19 Q Dr. Birrer, throughout your report you,</p> <p>20 at least at times, used the term "talc." What</p> <p>21 are you referring to when you say talc?</p> <p>22 A So there's two levels of relevance</p> <p>23 here. One is for epidemiologic studies or</p> <p>24 studies that were -- that were conducted. A</p>	<p style="text-align: right;">Page 84</p> <p>1 sense is they command the market. But I'm not --</p> <p>2 I'm not in the supermarket a lot.</p> <p>3 Q And not in the baby powder section?</p> <p>4 A No.</p> <p>5 Q And what is contained in the</p> <p>6 Johnson's -- in Johnson's baby powder, to your</p> <p>7 understanding?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Talc. And I know that's an issue</p> <p>11 that's come up in terms of are there other</p> <p>12 things. I mean, clearly there are other things</p> <p>13 that -- the product smells nice, so there must be</p> <p>14 some fragrance.</p> <p>15 MS. THOMPSON:</p> <p>16 Q Okay.</p> <p>17 A But I don't know of any -- first of</p> <p>18 all, I don't -- that's not my area of expertise.</p> <p>19 I've certainly never conducted any experiments</p> <p>20 and tried to figure out what's in it and -- and</p> <p>21 wouldn't consider myself an expert in the whole</p> <p>22 mineralogy issue.</p> <p>23 Q So that would be talc, the mineral. Do</p> <p>24 you have an opinion as to whether there is a such</p>
<p style="text-align: right;">Page 83</p> <p>1 subset of the -- of the studies that were</p> <p>2 conducted in the lab were actually dealing with</p> <p>3 talcum powder.</p> <p>4 But there are experiments in particular</p> <p>5 where individuals are using sigma-produced talc.</p> <p>6 So it's -- it's -- it's a bit of a mixture. But</p> <p>7 I think, in particular in the epi studies, a lot</p> <p>8 of them are just okay to use powder.</p> <p>9 Q So to -- to the extent both of us can,</p> <p>10 we can try to say whether we're referring to</p> <p>11 talcum powder or talc, as you described, so</p> <p>12 let's -- let's both try to do that, to the extent</p> <p>13 possible, because it can get confusing.</p> <p>14 A I completely concur.</p> <p>15 Q Okay. Okay. I'm glad we agree on</p> <p>16 that.</p> <p>17 Do you know what Johnson & Johnson's</p> <p>18 market share of the talcum powder product has</p> <p>19 been over the years?</p> <p>20 A I don't.</p> <p>21 Q If I told you it was 60 to 70 percent,</p> <p>22 would you have any basis to disagree with that</p> <p>23 number?</p> <p>24 A I actually wouldn't, because I -- my</p>	<p style="text-align: right;">Page 85</p> <p>1 thing as pure talc?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 A You know, my -- you know, my sense is</p> <p>5 in that some of the experiments where this</p> <p>6 product is actually bought not cosmetically, but</p> <p>7 I've seen references to sigma-produced talc, that</p> <p>8 that's a -- that's a purified form of it.</p> <p>9 MS. THOMPSON:</p> <p>10 Q And, so, by pure -- purified form, you</p> <p>11 would mean that it does not con- -- contain</p> <p>12 impurities; correct?</p> <p>13 A It would not contain something else.</p> <p>14 Q Would you consider it pure if it</p> <p>15 contained talc fibers?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A I don't -- I don't think I can answer</p> <p>19 that.</p> <p>20 MS. THOMPSON:</p> <p>21 Q So no opinion on -- on that issue.</p> <p>22 A Yeah.</p> <p>23 Q Are you familiar with the various</p> <p>24 grades of talc?</p>

22 (Pages 82 to 85)

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<p style="text-align: right;">Page 86</p> <p>1 A No.</p> <p>2 Q Do you have any knowledge regarding the</p> <p>3 particle size of Johnson's baby powder or Shower</p> <p>4 to Shower?</p> <p>5 A Again, that's a little bit outside my</p> <p>6 area of expertise. My understanding is, you</p> <p>7 know, talc ranges from 10 microns to larger</p> <p>8 sizes. But it's not something I systematically</p> <p>9 explored. Even the expert reports here that</p> <p>10 focused on the mineralogy, I looked at it but not</p> <p>11 in any great detail.</p> <p>12 Q And if you were told that there are</p> <p>13 also smaller particles than 10 microns, that</p> <p>14 wouldn't surprise you?</p> <p>15 A I think there's a range.</p> <p>16 Q Fair enough.</p> <p>17 A I don't know how -- you know, again, I</p> <p>18 know there's references to ultrafine, et cetera,</p> <p>19 et cetera. I don't have definitive knowledge or</p> <p>20 data that that is true.</p> <p>21 Q Okay. But, as far as you know, the</p> <p>22 particle size is -- is mixed?</p> <p>23 A Uh-huh.</p> <p>24 Q It's not a standard size like you might</p>	<p style="text-align: right;">Page 88</p> <p>1 Q It was the -- it was a report that</p> <p>2 addressed the fragrance chemicals in talcum</p> <p>3 powder. Do you remember seeing that? I don't</p> <p>4 remember whether it's on your list. Oh.</p> <p>5 A Is that plaintiff?</p> <p>6 Q You don't have Dr. Crowley's report.</p> <p>7 A Yeah.</p> <p>8 Q Did you know if there was a -- an</p> <p>9 expert report that specifically addressed the</p> <p>10 fragrance -- fragrance chemical presence in baby</p> <p>11 powder?</p> <p>12 A Not that I know of.</p> <p>13 Q So I -- I can assume that you don't</p> <p>14 know why you weren't provided Dr. Crowley's</p> <p>15 report?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A It's not on my list.</p> <p>19 MS. THOMPSON:</p> <p>20 Q Did you ask if anyone had looked at the</p> <p>21 actual chemicals in baby powder?</p> <p>22 A I didn't specifically go through that,</p> <p>23 no.</p> <p>24 Q It -- is it important for you to know</p>
<p style="text-align: right;">Page 87</p> <p>1 see, for example, in a pleurodesis talc?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 A I don't -- I can't say that.</p> <p>5 MS. THOMPSON:</p> <p>6 Q Okay.</p> <p>7 A But based on my rudimentary</p> <p>8 understanding of mineralogy here, that there's a</p> <p>9 range.</p> <p>10 Q Have you ever looked at the label on a</p> <p>11 bottle of baby powder?</p> <p>12 A I don't recall that.</p> <p>13 Q So you don't know what would be listed</p> <p>14 on the label?</p> <p>15 A No.</p> <p>16 Q But you're assuming it has some kind of</p> <p>17 fragrances in it?</p> <p>18 A I think that's a safe assumption. I</p> <p>19 have smelled it.</p> <p>20 Q Haven't we all.</p> <p>21 Did you read Dr. Crowley's report?</p> <p>22 Do you remember Dr. Crowley's report?</p> <p>23 A That's not coming to mind. Can -- do</p> <p>24 you have it?</p>	<p style="text-align: right;">Page 89</p> <p>1 the quality of talcum powder?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 A And how do you define "quality"?</p> <p>5 MS. THOMPSON:</p> <p>6 Q I -- I define "quality" as the absence</p> <p>7 of the amount and types of impurities.</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A How do you define "impurities"?</p> <p>11 MS. THOMPSON:</p> <p>12 Q Something that's not pure talc.</p> <p>13 A Okay. Again, I -- I'll come back to</p> <p>14 this theme. I think -- I didn't go down that</p> <p>15 road. It's not my area of expertise. But, more</p> <p>16 importantly, I was asked to sort of review the</p> <p>17 total data that suggested there might be a role</p> <p>18 for talc in ovarian cancer, regard- -- talcum</p> <p>19 powder, regardless of what's in it.</p> <p>20 So in that context, impurities,</p> <p>21 fragrance, heavy metals, it doesn't matter. We</p> <p>22 would see the data. So I felt pretty comfortable</p> <p>23 that that's the -- that's the important theme for</p> <p>24 my job.</p>

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<p style="text-align: right;">Page 90</p> <p>1 Q Is it important for you to know the</p> <p>2 min- -- mineral content of a talcum powder</p> <p>3 product if you are intending to assess its</p> <p>4 potential health effects?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A Would you just repeat that, please?</p> <p>8 MS. THOMPSON:</p> <p>9 Q Is it important to know the mineral</p> <p>10 content of a talcum powder product if you are</p> <p>11 intending to assess its potential health effects?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A You know, again, I think in terms of</p> <p>15 reviewing the literature, no. I mean, it's</p> <p>16 talcum and it's talcum powder. It's a</p> <p>17 representative of what's on the market.</p> <p>18 So regardless of what's there or not,</p> <p>19 even from a mineral standpoint, we can make a</p> <p>20 judgment as to whether that's providing data that</p> <p>21 supports whether it's a risk factor or biologic</p> <p>22 plausibility for a role in development of ovarian</p> <p>23 cancer.</p> <p>24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 92</p> <p>1 MS. THOMPSON:</p> <p>2 Q For a potential health effect.</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A There's no data for that. I can't</p> <p>6 develop a mechanism when, in fact, there's no</p> <p>7 biologic plausibility for talcum powder in a role</p> <p>8 of ovarian cancer.</p> <p>9 MS. THOMPSON:</p> <p>10 Q Well, it sounds like what you're saying</p> <p>11 is if you decide that talcum powder doesn't cause</p> <p>12 ovarian cancer, then there's no reason to even</p> <p>13 look at whether there's a plausible mechanism or</p> <p>14 not.</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 MS. THOMPSON:</p> <p>18 Q Is that --</p> <p>19 A Well, I'm not sure what mechanism we're</p> <p>20 looking at. We're looking at a mechanism that an</p> <p>21 agent doesn't cause cancer? That does -- makes</p> <p>22 no sense to me.</p> <p>23 Q We're looking at what a mechanism could</p> <p>24 be if it could cause cancer, as a hypothetical.</p>
<p style="text-align: right;">Page 91</p> <p>1 Q So even in your determination of</p> <p>2 whether a biologic mechanism is plausible or not,</p> <p>3 it doesn't matter what the mineral content of the</p> <p>4 baby powder is?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A As long as that baby powder's been</p> <p>8 tested in that experiment, it doesn't matter.</p> <p>9 MS. THOMPSON:</p> <p>10 Q And that goes for whether the baby</p> <p>11 powder contains asbestos?</p> <p>12 A Well, again, I -- I think if it</p> <p>13 contained asbestos, that would show a signal in</p> <p>14 those experiments. Now, we would see it. We may</p> <p>15 not know it's related to asbestos, fragrance or</p> <p>16 whatever, but the experiments would be</p> <p>17 reproducible and dispositive. And in my</p> <p>18 experience, they're not.</p> <p>19 Q But the question is, does that -- would</p> <p>20 that explain a mechanism if there's asbestos in</p> <p>21 the baby powder?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A Mechanism for what?</p>	<p style="text-align: right;">Page 93</p> <p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A No. I -- a mechanism for a</p> <p>4 hypothetical. I -- you know, again, that -- we</p> <p>5 don't need the hypothetical. We've tested talcum</p> <p>6 in those experiments. There's no data to support</p> <p>7 biologic plausibility. So why are -- why would</p> <p>8 we be trying to think about a hypothetical</p> <p>9 component to produce a mechanism for a biologic</p> <p>10 activity that we haven't seen?</p> <p>11 MS. THOMPSON:</p> <p>12 Q What experiments are you referring to?</p> <p>13 A I would say primarily the ones that are</p> <p>14 in my expert report. That really is a sum- --</p> <p>15 Q Which experiments in your report? We</p> <p>16 can go through your report if you want.</p> <p>17 A I'm -- yeah.</p> <p>18 Q I'm looking for the experiments that</p> <p>19 show that there's no biologic effect.</p> <p>20 A So Buz'Zard is one that frequently --</p> <p>21 Q And is it your opinion that Buz'Zard</p> <p>22 shows no biologic effect?</p> <p>23 A There's nothing in that paper that's</p> <p>24 reliable in terms of showing biologic</p>

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<p style="text-align: right;">Page 94</p> <p>1 plausibility.</p> <p>2 Q And we'll get to the others.</p> <p>3 So you're referring to --</p> <p>4 A Yes.</p> <p>5 Q -- Buz'Zard, Shukla?</p> <p>6 A Shukla. Just hang on. Yeah.</p> <p>7 Buz'Zard, Shukla and Hamilton.</p> <p>8 Q And I'm going to assume you include</p> <p>9 Dr. Saed in that?</p> <p>10 A Correct.</p> <p>11 Q Although we're going to get into more</p> <p>12 detail in that later.</p> <p>13 A Exactly.</p> <p>14 Q And you're aware of the other animal</p> <p>15 studies that show inflammatory effects; right?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A You have to go through those and define</p> <p>19 that.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Okay.</p> <p>22 A Because it's pretty broad literature.</p> <p>23 You're assuming -- you're referring to</p> <p>24 Keskin?</p>	<p style="text-align: right;">Page 96</p> <p>1 What is your understanding of how these</p> <p>2 products are used by women?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A Baby powder?</p> <p>6 MS. THOMPSON:</p> <p>7 Q And -- and we're talking about, at</p> <p>8 least for these cases, in the perineal area.</p> <p>9 A Yeah.</p> <p>10 Q Do you have any knowledge from</p> <p>11 conversations with women or literature or any</p> <p>12 other source as to how it's applied, whether it's</p> <p>13 standing, lying down, in the underwear, on a</p> <p>14 sanitary napkin, shaken into hands? Did you have</p> <p>15 any understanding of -- of those issues?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A I would say not a systematic, shall we</p> <p>19 say, meta-analysis of baby powder use. I</p> <p>20 certainly, over years in the clinic, am familiar</p> <p>21 with women who use baby powder. You know, my</p> <p>22 sense is that most dust the perineum usually</p> <p>23 standing up. I -- but again, I can't say that's</p> <p>24 a scientific evaluation. I have some experience</p>
<p style="text-align: right;">Page 95</p> <p>1 Q There are studies going back to the</p> <p>2 '40s and '50s with intraperitoneal inflammatory</p> <p>3 effects with -- in the presence of talc.</p> <p>4 You're aware of those?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A There is a big literature.</p> <p>8 MS. THOMPSON:</p> <p>9 Q And understanding that there are</p> <p>10 different histologic subtypes of epithelial</p> <p>11 ovarian cancer, can we agree that if one of us</p> <p>12 refers to ovarian cancer in a general sense, that</p> <p>13 we're referring to epithelial ovarian cancer?</p> <p>14 A I would not include germ -- you know,</p> <p>15 germ cell tumors in this.</p> <p>16 Q Stromal -- we're excluding stromal --</p> <p>17 A And stromal, yeah. It's epithelial,</p> <p>18 correct.</p> <p>19 Q Okay. So we're on the same page there?</p> <p>20 A With -- with the caveat being, and we</p> <p>21 do discuss this in the report about -- even</p> <p>22 within the epithelial component, we now realize</p> <p>23 there are different types of tumors.</p> <p>24 Q Understood.</p>	<p style="text-align: right;">Page 97</p> <p>1 with my wife. So I -- I -- it's a certain --</p> <p>2 some general concept of how it's done, yeah.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Would you agree, at least, that, for</p> <p>5 most women, it would be applied in a -- in a</p> <p>6 habitual manner?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Yeah, I think it's important to define</p> <p>10 that. It would certainly be repetitive. Is it</p> <p>11 something -- you know, habitual sounds to me</p> <p>12 like -- almost like an addict. And I don't -- I</p> <p>13 don't think that's the case.</p> <p>14 MS. THOMPSON:</p> <p>15 Q No. I didn't mean it -- mean in that</p> <p>16 term.</p> <p>17 I meant that it's -- and this has been</p> <p>18 reported in the literature, I believe you're</p> <p>19 aware --</p> <p>20 A Uh-huh.</p> <p>21 Q -- that most women do it the same way</p> <p>22 every day or whatever schedule they're on.</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>

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<p style="text-align: right;">Page 98</p> <p>1 A I would think that there'd be some 2 consistency on that. I -- I will say this 3 parenthetically, you may get to it later on, but 4 I do think, based on what we're just discussing, 5 it's very hard to -- it's very hard to quantify 6 amount of use. I really do. 7 MS. THOMPSON: 8 Q And I think we will get to that. 9 A Okay. 10 Q But -- but -- so it's hard to quantify 11 how much a woman is using on any given 12 application; correct? 13 A (Nods affirmatively.) 14 Q And it's hard -- 15 MS. CURRY: 16 You have to say "yes" or "no" versus 17 head shakes because the court reporter will not 18 be able to get that down. 19 A It says "nods affirmatively." 20 Yes. 21 MS. CURRY: 22 She was able to in that instance. I 23 stand corrected, but for -- 24 THE WITNESS:</p>	<p style="text-align: right;">Page 100</p> <p>1 be true for a number of environmental 2 exposures -- 3 MS. CURRY: 4 Object to the form. 5 MS. THOMPSON: 6 Q -- that difficulty in quantifying how 7 much a particular individual is exposed to? 8 A Well, you'd have to give me some 9 examples on that. I mean, I think for cigarette 10 smoke, it actually is quite quantifiable. 11 Q Cigarette smoke, I agree. 12 How about a household or domestic 13 exposure to asbestos, for example? 14 A I guess you could quantify the amount 15 of asbestos-containing material in the house, 16 but -- 17 Q How about a spouse coming home from 18 occupational exposure? 19 A Yeah. It would be a challenge. 20 Q How about chemicals in water source? 21 A That should be measurable. 22 Q Over time? 23 A Multiple samples. 24 Q How about --</p>
<p style="text-align: right;">Page 99</p> <p>1 She's very good. 2 MS. THOMPSON: 3 Q And -- and if there were talc that 4 reached the vagina or the upper genital tract, it 5 would be hard to quantify how much that would be; 6 right? 7 A Yes. 8 Q But you'll have to agree, but -- that 9 not being able to quantify it isn't a reason not 10 to study the issue. Right? 11 MS. CURRY: 12 Object to the form. 13 A I think that's a fair statement in 14 that, you know, if it's important, you need to do 15 it. I just think that, for the reasons you just 16 said, quantifying it is -- is difficult, not only 17 in individual applications, how much actually 18 would get where, but this longitudinal issue. 19 While I think there's some consistency, do women 20 use it for a while and then stop using it and how 21 often do they change? I think there's a whole 22 issue on that, too. 23 MS. THOMPSON: 24 Q And wouldn't you agree that that would</p>	<p style="text-align: right;">Page 101</p> <p>1 A And -- and potentially even the 2 patient. 3 Q How about exposure to a pesticide? 4 A Yeah. That would be more of a 5 challenge. Yeah. 6 Q So there's certainly other -- 7 A Some variability. 8 Q -- other situations where it's 9 challenging to quantify the exposure to an 10 individual over time. 11 MS. CURRY: 12 Object to the form. 13 A Yes. 14 MS. THOMPSON: 15 Q Other than a literature or document 16 review, you -- I think I asked you this before 17 but I'm gonna just ask it again since it's in my 18 outline here. 19 Other than a literature and document 20 review, have you done any research on talcum 21 powder and ovarian cancer? 22 A No. 23 Q And that would include in vitro 24 research and in vivo; correct?</p>

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<p>1 A Correct.</p> <p>2 Q And you've never published an article</p> <p>3 on talcum powder and ovarian cancer. Is that</p> <p>4 correct?</p> <p>5 A No.</p> <p>6 Q Have you ever given a talk on talcum</p> <p>7 powder and ovarian cancer?</p> <p>8 A No.</p> <p>9 Q Have you discussed your opinions in</p> <p>10 this case with anyone?</p> <p>11 A No, other than counsel.</p> <p>12 Q No colleagues?</p> <p>13 A No.</p> <p>14 Q Did you attend the recent SGO</p> <p>15 conference in Hawaii?</p> <p>16 A Hawaii's a nice place. I did.</p> <p>17 Q Did you discuss talcum powder with any</p> <p>18 of your colleagues at the meeting?</p> <p>19 A I'd never been there before.</p> <p>20 I did not.</p> <p>21 Q Do you know Liz Swisher?</p> <p>22 A I do know Liz, yes.</p> <p>23 Q Do you know her from professional</p> <p>24 meetings and other interactions?</p>	<p>1 Q Do you know why she's no longer an</p> <p>2 expert?</p> <p>3 A I don't.</p> <p>4 Q Do you know Dr. Huh?</p> <p>5 A I do know Dr. Huh. Warner. Uh-huh.</p> <p>6 Q Do you know why Dr. Huh is not serving</p> <p>7 as an expert for the defendants in the MDL?</p> <p>8 A No.</p> <p>9 Q Does University of Alabama know that</p> <p>10 you are serving as a paid expert for</p> <p>11 Johnson & Johnson --</p> <p>12 A Yes.</p> <p>13 Q -- in this case?</p> <p>14 Do you know how much money</p> <p>15 Johnson & Johnson has contributed to the</p> <p>16 University of Alabama and your lab?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A I --</p> <p>20 MS. THOMPSON:</p> <p>21 Q Let me rephrase that question because I</p> <p>22 don't like being "contributed."</p> <p>23 Do you know how much money</p> <p>24 Johnson & Johnson has paid to University of</p>
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<p>1 A I know her professionally and we're on</p> <p>2 several papers together.</p> <p>3 Q Yes, you are.</p> <p>4 A Yeah.</p> <p>5 Q Have you discussed the case with</p> <p>6 Dr. Swisher?</p> <p>7 A Not that I can recall.</p> <p>8 Q Were you aware that she was originally</p> <p>9 disclosed as an expert for the defendants?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A I think her name did -- was sort of</p> <p>13 mentioned to me, but --</p> <p>14 MS. CURRY:</p> <p>15 And please don't reveal any discussions</p> <p>16 or --</p> <p>17 THE WITNESS:</p> <p>18 Okay.</p> <p>19 MS. CURRY:</p> <p>20 -- communications that you've had with</p> <p>21 lawyers.</p> <p>22 THE WITNESS:</p> <p>23 Yes, counsel.</p> <p>24 MS. THOMPSON:</p>	<p>1 Alabama?</p> <p>2 A No.</p> <p>3 Q Do you know how much money</p> <p>4 Johnson & Johnson has paid to support your lab?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A None.</p> <p>8 MS. CURRY:</p> <p>9 We've been going over an hour and a</p> <p>10 half. Whenever it's a good breaking point for</p> <p>11 you.</p> <p>12 MS. THOMPSON:</p> <p>13 I think maybe less than five minutes --</p> <p>14 MS. CURRY:</p> <p>15 No problem.</p> <p>16 MS. THOMPSON:</p> <p>17 -- and it's a great break time.</p> <p>18 A I may be in kidney failure soon.</p> <p>19 MS. THOMPSON:</p> <p>20 Q Can you make five minutes?</p> <p>21 A Yeah, I can. Yeah.</p> <p>22 Q We'll -- we'll --</p> <p>23 A Sure.</p> <p>24 Q -- be in the same boat there, so we</p>

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<p>1 can --</p> <p>2 A Boat's not a good choice.</p> <p>3 Q Yeah. I should have used a different</p> <p>4 word there.</p> <p>5 We talked about the methodology that</p> <p>6 you applied, but -- but it's not included, per</p> <p>7 se, in the report.</p> <p>8 Can you refer to me -- me to any</p> <p>9 published article, textbook chapter, anything</p> <p>10 that actually describes Dr. Birrer's methodology?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A No. Again, I -- I think this relates</p> <p>14 to what a lot of us in the field on my level do</p> <p>15 routinely, and so it's not really defined. But</p> <p>16 when we review literature, a topic, I wouldn't</p> <p>17 want to -- I don't want to call it a</p> <p>18 meta-analysis because that's a formal process.</p> <p>19 But we -- we -- we do the right -- we do the same</p> <p>20 thing. If we do it right, then it's</p> <p>21 comprehensive and then we make opinions on those</p> <p>22 papers. That's the methodology.</p> <p>23 MS. THOMPSON:</p> <p>24 Q Okay.</p>	<p>1 MS. THOMPSON:</p> <p>2 Q How about what is sometimes used in the</p> <p>3 literature, elongated mineral fibers? Does that</p> <p>4 sound familiar?</p> <p>5 A It sounds consistent with some of the</p> <p>6 things I read, but I certainly did not pursue</p> <p>7 that sort of mineralogy review.</p> <p>8 Q So no comprehensive review on what's</p> <p>9 called EMP sometimes.</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A No.</p> <p>13 MS. THOMPSON:</p> <p>14 Q And I can assume that you didn't do a</p> <p>15 comprehensive review on heavy metals --</p> <p>16 A Correct.</p> <p>17 Q -- and ovarian cancer?</p> <p>18 A Yes.</p> <p>19 Q Or fragrance chemicals and ovarian</p> <p>20 cancer?</p> <p>21 A Correct.</p> <p>22 Q Do you agree that scientists can look</p> <p>23 at the same body of literature and reach</p> <p>24 different conclusions, in a general sense?</p>
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<p>1 A It's more of a scientific lab-based</p> <p>2 approach.</p> <p>3 Q Okay. And did you apply the same</p> <p>4 standards for this report that you would use if</p> <p>5 you were publishing a paper, for example, a</p> <p>6 review article like we discussed before?</p> <p>7 A I think so, yes.</p> <p>8 Q Would you be willing to have the</p> <p>9 opinions that you've provided in this report</p> <p>10 peer-reviewed if that were appropriate?</p> <p>11 A Essentially, yes. Yeah. Yeah.</p> <p>12 Q And I think we've discussed this, but</p> <p>13 does -- in your opinion, you performed a</p> <p>14 comprehensive literature review on the subject of</p> <p>15 talc and ovarian cancer; correct?</p> <p>16 A Correct.</p> <p>17 Q But am I correct to say that you did</p> <p>18 not perform the same comprehensive literature</p> <p>19 review for asbestos and ovarian cancer?</p> <p>20 A Correct.</p> <p>21 Q Fibrous talc in ovarian cancer?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A Didn't use that term.</p>	<p>1 A You know, again, I think if the body</p> <p>2 of -- of data and literature is substantive and</p> <p>3 clear, I think that a reasonable scientist, a</p> <p>4 competent scientist will come to the same</p> <p>5 conclusion.</p> <p>6 Q So is it your opinion that a scientist</p> <p>7 who looks at the baby powder literature or talcum</p> <p>8 powder literature and concludes something</p> <p>9 different from you is unreasonable and</p> <p>10 incompetent?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A I -- I would say they got it wrong.</p> <p>14 MS. THOMPSON:</p> <p>15 Q They got it wrong. But what about</p> <p>16 unreasonable?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A I don't -- I wouldn't use that term. I</p> <p>20 would say that they looked at the data and</p> <p>21 misinterpreted it.</p> <p>22 MS. THOMPSON:</p> <p>23 Q And would you say the same about their</p> <p>24 competence?</p>

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<p style="text-align: right;">Page 110</p> <p>1 MS. CURRY: 2 Object to the form. 3 A I think -- you know, labeling that as 4 incompetent is not appropriate. 5 MS. THOMPSON: 6 Q Well, you said, I think that a 7 reasonable scientist, competent scientist will 8 come to the same conclusion. Wouldn't that imply 9 that if they come to a different inclusion -- 10 conclusion, that they're unreasonable or 11 incompetent? 12 A Well, I think I prefaced that with if 13 the body of science we're looking at is -- is -- 14 it's convincing and strong and reproducible, that 15 reasonable scientists will come to the same 16 conclusion. 17 When the data is really unconvincing, 18 which is what we're dealing with here -- this 19 data is not convincing -- there's no data for 20 talc being involved in ovarian cancer, then you 21 get this disparate opinions. And -- and they've 22 got it wrong. And I made the -- 23 Q They've got it -- sorry. 24 A And I've made the argument why I got it</p>	<p style="text-align: right;">Page 112</p> <p>1 A Okay. 2 MS. CURRY: 3 Can we take a break? 4 A It looks like you're coming to an end. 5 MS. THOMPSON: 6 Q We are. Well, not the end of the day. 7 The end of the section. 8 A Hope springs eternal. 9 Q Wishful thinking. 10 One -- one more question, then we're 11 done. 12 A Sure. 13 Q What does "proof" mean to you? 14 MS. CURRY: 15 Object to the form. 16 MS. THOMPSON: 17 Q In a scientific sense. 18 A That would be evidence to support the 19 conclusion. 20 Q To convincingly support the conclusion? 21 MS. CURRY: 22 Object to the form. 23 A I'm not sure I need that adjective 24 there.</p>
<p style="text-align: right;">Page 111</p> <p>1 right. 2 Q Okay. They've got it wrong? 3 A Uh-huh. 4 Q You have it right. 5 A Uh-huh. 6 Q But I'm trying to find -- figure out 7 how you think they got it wrong. Were they 8 misinformed? 9 MS. CURRY: 10 Object to the form. 11 A They misinterpreted the data. 12 MS. THOMPSON: 13 Q They misinterpreted the data. 14 A Yeah. 15 Q And you would say they misinterpreted 16 the data even though they interpreted the data in 17 the same way that the authors presenting the data 18 pre- -- interpreted it? 19 MS. CURRY: 20 Object to the form. 21 A We'd have to go through the actual 22 paper you're referring to. 23 MS. THOMPSON: 24 Q Okay. We may go through some of those.</p>	<p style="text-align: right;">Page 113</p> <p>1 MS. THOMPSON: 2 Q Well, support -- support equals proof? 3 A Support couldn't equal proof. Proof is 4 a general term. So it's gonna be a spectrum. 5 Q 100 percent? 6 A Are you -- you know, definitive proof 7 would be definitive. 8 Q Okay. Let's take a break. 9 VIDEOGRAPHER: 10 Off the record at 10:44 a.m. 11 (OFF THE RECORD.) 12 VIDEOGRAPHER: 13 We're back on the record at 11 a.m. 14 MS. THOMPSON: 15 Q Dr. Birrer, I want to give you a series 16 of statements and have you agree or disagree or, 17 if you don't know or don't have an opinion, 18 that's fine, too. And -- and if you do have a 19 comment or explanation, you're welcome to provide 20 that, too, after you -- do you have a pen? You 21 can mark on this exhibit as we go through. This 22 is Exhibit 9. 23 (DEPOSITION EXHIBIT NUMBER 9 24 WAS MARKED FOR IDENTIFICATION.)</p>

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<p style="text-align: right;">Page 114</p> <p>1 MS. CURRY: 2 Can I just state an objection on the 3 record to the creation of this exhibit without 4 knowing the background of where the statements 5 are coming from. 6 MS. GARBER: 7 I don't think we're going to have 8 speaking objections here today, Miss Curry. The 9 proper objection is "Objection. Form." Do not 10 coach the witness, please. 11 MS. CURRY: 12 Miss Garber, I'm not coaching the 13 witness. 14 MS. GARBER: 15 You are coaching the witness. You know 16 you're coaching the witness. 17 MS. THOMPSON: 18 I'm asking a statement. It doesn't 19 matter where it's coming from. It's from my 20 head. 21 MR. MIZGALA: 22 Do you have extra copies of this? 23 MS. THOMPSON: 24 I did bring extra copies.</p>	<p style="text-align: right;">Page 116</p> <p>1 A Yeah. I would disagree with that 2 statement. 3 Q Number 2, "If 40 percent of women use 4 talc and the relative risk is 1.2, then 7 percent 5 of ovarian cancer cases would be attributable to 6 talc use or 1,577 cases a year in the USA. This 7 is not a trivial number and should not be 8 dismissed." 9 Would you agree or disagree? 10 MS. CURRY: 11 Object to the form. 12 A Disagree. 13 MS. THOMPSON: 14 Q Number 3, "Genital powder use is a 15 modifiable exposure associated with small to 16 moderate increases in risk of most histologic 17 subtypes of epithelial ovarian cancer." 18 Would you agree or disagree? 19 MS. CURRY: 20 Object to the form. 21 A Disagree. 22 I'm sorry. Go ahead. Got it? 23 Disagree. 24 MS. THOMPSON:</p>
<p style="text-align: right;">Page 115</p> <p>1 MR. MIZGALA: 2 Thank you. 3 MS. THOMPSON: 4 Q So, Dr. Birrer, statement number 1, 5 "Given the number of hazard ratios reported in 6 the literature between 1.1 and" -- that should be 7 an -- "1.4 in both case-control and cohort 8 studies, it is disingenuous to state that there 9 is no evidence that talc is associated with 10 ovarian cancer." 11 Do you agree or disagree with that 12 statement? 13 MS. CURRY: 14 Object to the form. 15 A Now, you want me to write an answer 16 here? 17 MS. THOMPSON: 18 Q Yes, please. And then -- and when you 19 tell me, I'm going to put it on here, too. 20 A Yeah. Okay. In these -- the hazard 21 ratios, these are in a case-controlled cohort 22 studies. 23 Q It says in both case-controlled and 24 cohort studies.</p>	<p style="text-align: right;">Page 117</p> <p>1 Q Number 4, "Perineal use of talc-based, 2 not asbestiform, body powder is possibly 3 carcinogenic to humans, group 2B." 4 A Disagree. 5 MS. CURRY: 6 Object to the form. 7 MS. THOMPSON: 8 Q Number 5, "The use of perineal talcum 9 powder has been associated with a 20 to 30 10 percent increased risk of ovarian cancer, 11 although it also has been shown to vary by 12 histologic subtype." 13 MS. CURRY: 14 Object to the form. 15 MS. THOMPSON: 16 Q Agree or disagree? 17 A And this is -- like, histologic -- 18 clear cell and endometrioid? Is that what's 19 being implied here? 20 Q Yes. 21 A Disagree. 22 Q Number 6, "A lot of work has been done 23 to clarify the risk reduction of various 24 lifestyle approaches, such as alcohol, obesity,</p>

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<p>1 cigarette smoking and talc use. Some of these</p> <p>2 are subtype specific, such as endometriosis,</p> <p>3 cigarette smoking, while others are general risk</p> <p>4 factors. Use of talc in the genital area has</p> <p>5 consistently been shown to increase the risk of</p> <p>6 OC and therefore is not recommended."</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Disagree.</p> <p>10 MS. THOMPSON:</p> <p>11 Q Number 7, "Inflammatory risk factors</p> <p>12 for EOC are perineal talc exposure, endometriosis</p> <p>13 and pelvic inflammatory disease."</p> <p>14 Agree or disagree?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A So this is inclusive of all three;</p> <p>18 right? Endometriosis and --</p> <p>19 MS. THOMPSON:</p> <p>20 Q Yes.</p> <p>21 A Okay.</p> <p>22 Q But if you want to disagree and</p> <p>23 explain, that -- that's fine.</p> <p>24 A I would -- that's a tough one to</p>	<p>1 statement as a whole --</p> <p>2 A Yeah.</p> <p>3 Q -- but would --</p> <p>4 A Caveat.</p> <p>5 Q -- and that will be on the record that</p> <p>6 you --</p> <p>7 A Okay. Parsed it.</p> <p>8 Q The ones that -- yeah.</p> <p>9 Number 9, "Talc powder use is highly</p> <p>10 prevalent in the African-American community and</p> <p>11 has been found to be associated with increased</p> <p>12 risk of ovarian cancer, period."</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A So I do believe the first part, that</p> <p>16 it's prevalent in the African-American community.</p> <p>17 The second part is not convincing to me.</p> <p>18 Is that -- can we put that on the</p> <p>19 record? Disagree with the caveat, yeah.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Yeah. "Most women report using</p> <p>22 Johnson's baby powder or Shower to Shower."</p> <p>23 A I don't know.</p> <p>24 Q "The average age women begin using talc</p>
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<p>1 answer. I think endometriosis is a -- I don't</p> <p>2 call it inflammatory. So, yeah, I would -- I</p> <p>3 don't call it inflammatory, so, yeah, I would</p> <p>4 disagree on this. It's too general.</p> <p>5 MS. THOMPSON:</p> <p>6 Q "Risk factors to be considered:</p> <p>7 Parity, oral contraceptive use, breastfeeding,</p> <p>8 tubal ligation, painful periods or endometriosis,</p> <p>9 obesity or polycystic ovarian syndrome, and talc</p> <p>10 use. These risk factors are concordant with</p> <p>11 published epidemiologic data related to</p> <p>12 reproductive factors, use of talc, tubal</p> <p>13 ligation, endometriosis and polycystic ovarian</p> <p>14 syndrome or obesity."</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A So parity, oral contraceptive,</p> <p>18 breastfeeding, tubal ligation, endometriosis but</p> <p>19 not painful periods or obesity or talc use. Is</p> <p>20 that a --</p> <p>21 MS. THOMPSON:</p> <p>22 Q Okay.</p> <p>23 A -- no or --</p> <p>24 Q So -- so you would disagree with the</p>	<p>1 is 20."</p> <p>2 A Don't know that.</p> <p>3 Q "In the interest of public health, I</p> <p>4 believe we should caution women against using</p> <p>5 genital talcum powder," number 12.</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 MS. THOMPSON:</p> <p>9 Q Agree or disagree?</p> <p>10 A I disagree.</p> <p>11 Q Number 13, "Genital powder use is a</p> <p>12 lifestyle risk factor for all serous,</p> <p>13 endometrioid, and clear cell histologic subtypes</p> <p>14 of ovarian cancer."</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A I disagree.</p> <p>18 MS. THOMPSON:</p> <p>19 Q Number 14, "Overall, there is an</p> <p>20 association between genital talc use and EOC and</p> <p>21 a significant trend with increasing" -- in</p> <p>22 quotations -- "talc years of use."</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>

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<p>1 MS. THOMPSON: 2 Q Agree or disagree? 3 A I'm thinking. Disagree. 4 Q Number 15, "Talc-containing powders are 5 hypothesized to promote cancer development by 6 ascending the female genital tract and 7 interacting directly with the ovarian surface 8 epithelium, leading to local inflammation 9 characterized by increased rates of cell 10 division, DNA repair, oxidative stress, and 11 elevated inflammatory cytokines." 12 MS. CURRY: 13 Object to the form. 14 A This is a hypothesis; right? 15 MS. THOMPSON: 16 Q Yes. 17 A I agree. 18 Q "Following" -- number 16. 19 A Uh-huh. 20 Q "Following perineal application, talc 21 particles can migrate from the vagina to the 22 peritoneal cavity and ovaries." 23 MS. CURRY: 24 Object to the form.</p>	<p>1 present in the vagina, can migrate to the upper 2 genital tract." 3 MS. CURRY: 4 Object to the form. 5 MS. THOMPSON: 6 Q Agree or disagree? 7 MS. THOMPSON: 8 A You want to -- do you want to define 9 "biologic credibility"? 10 THE COURT REPORTER: 11 Say again? 12 THE WITNESS: 13 Define "biologic credibility." 14 Sorry. I'm mumbling. 15 THE COURT REPORTER: 16 Uh-huh. 17 MS. THOMPSON: 18 Q Let's define it as evidence of a 19 credible biologic mechanism. 20 A I would disagree. 21 MS. CURRY: 22 Object to the form. 23 MS. THOMPSON: 24 Q Number 20, "The vagina serves as a</p>
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<p>1 A Disagree on that. 2 MS. THOMPSON: 3 Q Number 17, "A majority of women 4 experience retrograde menstruation. This 5 suggests a mechanism by which talc particles can 6 travel through the female reproductive tract to 7 the peritoneal cavity and ovaries." 8 MS. CURRY: 9 Object to the form. 10 MS. THOMPSON: 11 Q Agree or disagree? 12 A Disagree. 13 Q Number 18, "It is possible that the 14 passage of talc is aided by retrograde menses and 15 that talc use during menses poses a special 16 risk." 17 Agree or disagree? 18 MS. CURRY: 19 Object to the form. 20 A Disagree. 21 MS. THOMPSON: 22 Q 19, "Biologic credibility of the 23 Talc/EOC association is enhanced by persuasive 24 evidence that inert particles the size of talc,</p>	<p>1 portal to the internal reproductive tract. 2 MS. CURRY: 3 Object to the form. 4 A Agree. 5 MS. THOMPSON: 6 Q 21, "The vagina is a musculoepithelial 7 tube extending from the level of the external 8 genitals to the cervical portion of the uterus. 9 It is a reproductive conduit in all respects, 10 connecting the external environment to the 11 internal genitalia." 12 MS. CURRY: 13 Object to the form. 14 A I'm not sure I understand that 15 statement. 16 What's the internal genitalia? 17 MS. THOMPSON: 18 Q The ovaries. 19 A The ovaries. I'm putting that in here. 20 Q And tubes. Let's say tubes and 21 ovaries. 22 A Okay. External. 23 Yeah, I would agree on that. 24 Q And, actually, I think the --</p>

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<p>1 A Cervix.</p> <p>2 Q I think the uterus is an internal</p> <p>3 genitalia, too.</p> <p>4 A Okay.</p> <p>5 Q But I agree that's somewhat --</p> <p>6 A Yeah. It's a little -- I mean, yeah.</p> <p>7 Genitalia is usually external.</p> <p>8 Q Yeah.</p> <p>9 22, "A review of the literature</p> <p>10 suggests that it is biologically plausible for</p> <p>11 talc particles to migrate from the vagina to the</p> <p>12 peritoneal cavity and ovaries following perineal</p> <p>13 application."</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Agree or disagree?</p> <p>18 A Disagree.</p> <p>19 Q "Talc" -- 23. "Talc placed on the</p> <p>20 perineum may enter the vagina and ascend to the</p> <p>21 upper genital tract."</p> <p>22 Agree or disagree?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p>1 A Disagree.</p> <p>2 MS. THOMPSON:</p> <p>3 Q 27, "Talc is able to migrate through</p> <p>4 the genital tract and gain access to the ovaries</p> <p>5 because talc fibers have been detected in benign</p> <p>6 and malignant ovarian tissues."</p> <p>7 Agree or disagree?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Disagree.</p> <p>11 MS. THOMPSON:</p> <p>12 Q 28, "There are inherent limitations</p> <p>13 quantifying a dose-response due to a lack of</p> <p>14 metrics for how much talc is in an application,</p> <p>15 how much enters the vagina, and how much reaches</p> <p>16 the upper genital tract where, presumably, any</p> <p>17 deleterious effect is mediated. This may account</p> <p>18 for the failure to identify a dose-response in</p> <p>19 many papers on talc and ovarian cancer."</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A It's a big statement. Give me a</p> <p>23 second. I disagree with that.</p> <p>24 MS. THOMPSON:</p>
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<p>1 A Disagree.</p> <p>2 MS. THOMPSON:</p> <p>3 Q 24, "The potential for particulates to</p> <p>4 migrate from the perineum and vagina to the</p> <p>5 peritoneal cavity is indisputable."</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A Disagree.</p> <p>9 MS. THOMPSON:</p> <p>10 Q "The Sjösten study" --</p> <p>11 Do you know the Sjösten study?</p> <p>12 A I do.</p> <p>13 Q -- "offers compelling evidence in</p> <p>14 support of the migration hypothesis."</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A Disagree.</p> <p>18 MS. THOMPSON:</p> <p>19 Q 26, "Talc particulates from perineal</p> <p>20 application have been shown to migrate to the</p> <p>21 ovaries."</p> <p>22 Agree or disagree?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p>1 Q 29, "Tubal ligation is a strong</p> <p>2 protective factor. One possibility for the</p> <p>3 mechanism is blocking the transience of potential</p> <p>4 materials that could impact the health of the</p> <p>5 fimbria."</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A Disagree.</p> <p>9 MS. THOMPSON:</p> <p>10 Q Number 30, "Any material -- whether it</p> <p>11 be talc, heavy metals, asbestos, whatever -- can</p> <p>12 migrate from the perineum to the ovaries through</p> <p>13 the reproductive tract. There's an anatomical</p> <p>14 conduit, so it's not blocked. Theoretically, it</p> <p>15 could happen."</p> <p>16 Agree or disagree?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A Disagree.</p> <p>20 MS. THOMPSON:</p> <p>21 Q 31, "There is an anatomic conduit from</p> <p>22 the perineum through to the ovary, vagina,</p> <p>23 cervical os, endometrium, and the fallopian tube</p> <p>24 that is, in most women, an open conduit -- that</p>

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<p style="text-align: right;">Page 130</p> <p>1 is in most women an open conduit. On a theoretic 2 level, things can transit." 3 A I would agree with that. 4 MS. CURRY: 5 Object to the form. Sorry. 6 THE WITNESS: 7 I'm sorry. 8 MS. THOMPSON: 9 Q 32, "Genital powder use was associated 10 with ovarian cancer risk in African-American 11 women and are consistent with localized chronic 12 inflammation in the ovary due to particulates 13 that travel through a direct transvaginal route." 14 MS. CURRY: 15 Object to the form. 16 A Disagree. 17 MS. THOMPSON: 18 Q 33, "Biologic credibility for an 19 association would be strengthened by an animal 20 model, but an experiment capturing all of the 21 potential factors in the 'human' model would be 22 very difficult. These elements include 23 chronicity of the exposure, anatomic and 24 physiologic uniqueness of women, effects of</p>	<p style="text-align: right;">Page 132</p> <p>1 Oh, sorry. 2 So the animal model, yes. The rest of 3 it, no. 4 Q Animal model -- 5 A Would be strengthened. 6 Q Okay. We've got in the human model -- 7 A Yeah. 8 Q -- agree. 9 A Okay. 10 Q Okay. And the rest, disagree. 11 A Yeah. 12 Q Okay. I think that's clear, especially 13 with explanation. 14 34, "It is plausible that perineal 15 talc, and other particulate, in parens, that 16 reaches the endometrial cavity, fallopian tubes, 17 ovaries and peritoneum, may elicit a foreign 18 body-type reaction and inflammatory response 19 that, in some exposed women, may progress to 20 epithelial cancers." 21 MS. CURRY: 22 Object to the form. 23 A I disagree with that. 24 MS. THOMPSON:</p>
<p style="text-align: right;">Page 131</p> <p>1 pregnancy and potential spread through coitus." 2 Agree or disagree? 3 MS. CURRY: 4 Object to the form. 5 A This is in relationship to talc? 6 MS. THOMPSON: 7 Q Yes. 8 A Okay. 9 Q Talc and ovarian cancer. 10 A Yeah, yeah. Okay. 11 It's a two-part issue, unfortunately. 12 I mean, I think it would be strengthened by an 13 animal model. 14 Q And if you -- if you'd -- if you'd like 15 to divide that up into two sections, that would 16 be -- that's fine. 17 A Okay. Well, I -- okay. That's -- 18 yeah. I think -- I think it would be 19 strengthened by an animal model. 20 Q Okay. So -- 21 A "Experiment capturing all the potential 22 would be difficult." 23 I don't agree with that, the second 24 part. Can I do that and split it a little bit?</p>	<p style="text-align: right;">Page 133</p> <p>1 Q 35, "Epidemiologic evidence implicates 2 chronic inflammation as a central mechanism in 3 the pathogenesis of ovarian cancer, the most 4 lethal gynecologic cancer among women in the 5 United States." 6 MS. CURRY: 7 Object to the form. 8 MS. THOMPSON: 9 Q And I'll assume that you don't agree 10 with the last -- 11 A Right. Most lethal? 12 Q -- part of that? But the first part? 13 A I would disagree with this. Yeah. 14 Q 36, "Findings on talc and endometriosis 15 are consistent with previous findings and are 16 compatible with a hypothesis that these factors 17 increase the risk of ovarian cancer and that 18 inflammation -- and that inflammation may be a 19 common pathway." 20 MS. CURRY: 21 Object to the form. 22 A Disagree. 23 MS. THOMPSON: 24 Q 37, "Chron-" --</p>

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<p style="text-align: right;">Page 134</p> <p>1 A 37. Right.</p> <p>2 Q "Chronic inflammation has been proposed</p> <p>3 as the possible causal mechanism that explains</p> <p>4 the observed association between certain risk</p> <p>5 factors, such as use of talcum powder (talc) in</p> <p>6 the pelvic region and epithelial ovarian cancer."</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A That's been proposed; right? I would</p> <p>10 agree.</p> <p>11 MS. THOMPSON:</p> <p>12 Q And you would disagree that that is a</p> <p>13 possible cause of mechanism, I assume.</p> <p>14 A Correct.</p> <p>15 Q 38, "Talc particles can induce an</p> <p>16 inflammatory response in vivo, which may be</p> <p>17 important in ovarian cancer risk. Normal ovarian</p> <p>18 cells treated with talc are more likely to</p> <p>19 undergo cell proliferation and neoplastic</p> <p>20 transformation, and cellular generation of</p> <p>21 reactive oxygen species increases with increasing</p> <p>22 exposure to talc."</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p style="text-align: right;">Page 136</p> <p>1 inflammation and an increased risk of ovarian</p> <p>2 cancer. Other specific inflammatory factors have</p> <p>3 also been associated with ovarian cancer."</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 A I agree on that.</p> <p>7 MS. THOMPSON:</p> <p>8 Q 42, "The patency of the female tract</p> <p>9 and the nature of ovarian cancer as a surface</p> <p>10 epithelial (mesothelial lesion) make the ovary a</p> <p>11 target for foreign body carcinogenesis."</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Agree or disagree?</p> <p>16 A Disagree.</p> <p>17 Q 43, "Inflammation has been suggested to</p> <p>18 be a major factor leading to epithelial ovarian</p> <p>19 cancer. For example, epidemiologic data have</p> <p>20 shown that asbestos and talc exposure increased</p> <p>21 ovarian cancer risk."</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A Disagree.</p>
<p style="text-align: right;">Page 135</p> <p>1 A I disagree with that.</p> <p>2 MS. THOMPSON:</p> <p>3 Q 39, "A growing body of epidemiologic</p> <p>4 evidence suggests that factors causing epithelial</p> <p>5 inflammation are involved in ovarian</p> <p>6 carcinogenesis. Such factors include asbestos</p> <p>7 and talc exposures, endometriosis and pelvic</p> <p>8 inflammatory disease (PID)."</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A Disagree with that.</p> <p>12 MS. THOMPSON:</p> <p>13 Q 40, "Direct induction of inflammation</p> <p>14 as a result of endometriosis, talc, and asbestos</p> <p>15 exposure, and PID, as well as ovulation itself,</p> <p>16 may act to promote ovarian tumorigenesis."</p> <p>17 Agree or disagree?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A Disagree.</p> <p>21 MS. THOMPSON:</p> <p>22 Q 41, regarding Inflammation. "Studies</p> <p>23 of the inflammatory marker C-reactive protein</p> <p>24 suggests a possible association between</p>	<p style="text-align: right;">Page 137</p> <p>1 MS. THOMPSON:</p> <p>2 Q 44, "Studies have found" -- "also found</p> <p>3 that endometrio-" --</p> <p>4 Let's leave out the "also," since I</p> <p>5 don't know what that refers to.</p> <p>6 "Studies have found that endometriosis,</p> <p>7 pelvic inflammatory disease, and mumps viral</p> <p>8 infection are positively associated with ovarian</p> <p>9 cancer risk. In contrast, tubal ligations and</p> <p>10 hysterectomies, which are thought to reduce the</p> <p>11 exposure of the OSE to environmental inflammation</p> <p>12 initiators have been shown to reduce the risk of</p> <p>13 ovarian cancer."</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 A I agree on that.</p> <p>17 MS. THOMPSON:</p> <p>18 Q 45, "It has been noted that the</p> <p>19 ovulatory process itself resembles an</p> <p>20 inflammatory reaction, with leukocytic</p> <p>21 infiltration, the release of nitric oxide and</p> <p>22 inflammatory cytokines, basal dilation, DNA</p> <p>23 repair and tissue remodeling."</p> <p>24 MS. CURRY:</p>

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<p>1 Object to the form.</p> <p>2 MS THOMPSON:</p> <p>3 Q Agree or disagree?</p> <p>4 A I would agree on that.</p> <p>5 Q 46, "The latency period of more</p> <p>6 advanced, malignant epithelial ovarian cancer</p> <p>7 could be estimated to be approximately 30 to 40</p> <p>8 years."</p> <p>9 MS. CURRY:</p> <p>10 Form.</p> <p>11 A I don't know that. Sorry. I don't</p> <p>12 know.</p> <p>13 MS. THOMPSON:</p> <p>14 Q "If the magnitude of the association is</p> <p>15 to be estimated with precision, it is important</p> <p>16 that consortia are developed and expanded in</p> <p>17 order to generate the appropriate sample size."</p> <p>18 And this is in regard to talcum powder</p> <p>19 in association with ovarian cancer.</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A Don't know.</p> <p>23 MS. THOMPSON:</p> <p>24 Q 48, "Neither prospective study" --</p>	<p>1 Q 51, "For baby powder users, it is habit</p> <p>2 that developed at one point and stays regularly."</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A Don't know.</p> <p>6 MS. THOMPSON:</p> <p>7 Q 52, "In order to achieve statistical</p> <p>8 significance in a prospective study, we need a</p> <p>9 much larger cohort. For example, we will need to</p> <p>10 study upwards of 200,000 women for ten years."</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A I disagree.</p> <p>14 MS. THOMPSON:</p> <p>15 Q You disagree.</p> <p>16 53, "Given inherent limitation of</p> <p>17 cohort studies, it is not surprising that we have</p> <p>18 not been able to confirm the case-control studies</p> <p>19 with prospective studies, but this does not mean</p> <p>20 that the case-control studies were wrong."</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A Disagree.</p> <p>24 MS. THOMPSON:</p>
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<p>1 meaning Gertig or Houghton -- "confirmed the</p> <p>2 association of talc use and ovarian cancer raised</p> <p>3 by the case-control studies, but neither study</p> <p>4 was powered to detect a risk of 1.2 and</p> <p>5 therefore, we cannot exclude the possibility."</p> <p>6 Agree or disagree?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Disagree.</p> <p>10 MS. THOMPSON:</p> <p>11 Q 49, "An odds ratio of 1.2 or 1.3 has no</p> <p>12 meaningful clinical impact on a patient."</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A Don't know.</p> <p>16 MS. THOMPSON:</p> <p>17 Q "There are design studies with" --</p> <p>18 sorry.</p> <p>19 50, "There are design issues with every</p> <p>20 study, both case-controls and cohort studies."</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A I would agree with that.</p> <p>24 MS. THOMPSON:</p>	<p>1 Q Agree or disagree?</p> <p>2 A Disagree.</p> <p>3 Q 54, "It is unlikely that the</p> <p>4 association between talc and ovarian cancer is</p> <p>5 due to confounding, and so it is fair to say that</p> <p>6 if there is a statistically robust relationship</p> <p>7 between talc use and ovarian cancer" -- sorry.</p> <p>8 I'm gonna start all over.</p> <p>9 "It is unlikely that the association</p> <p>10 between talc and ovarian cancer is due to</p> <p>11 confounding, and so it is fair to say that if</p> <p>12 there is a statistically robust relationship</p> <p>13 between talc use and ovarian cancer, it is likely</p> <p>14 to be causal (albeit with intermediate factors</p> <p>15 such as inflammation)."</p> <p>16 Agree or disagree?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A Disagree.</p> <p>20 MS. THOMPSON:</p> <p>21 Q 55, "Among many epidemiologic</p> <p>22 variables, no confounders for the association --</p> <p>23 for the association were identified."</p> <p>24 MS. CURRY:</p>

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<p>1 Object to the form.</p> <p>2 A No opinion.</p> <p>3 MS. THOMPSON:</p> <p>4 Q 56, "There is a consistent association</p> <p>5 between talc and ovarian cancer that appears</p> <p>6 unlikely to be explained by recall or</p> <p>7 confounding."</p> <p>8 Agree or disagree?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A Disagree.</p> <p>12 MS. THOMPSON:</p> <p>13 Q 57, "The meta-analyses of the available</p> <p>14 human studies in the peer-reviewed literature</p> <p>15 indicate a consistent and statistically</p> <p>16 significant positive association between perineal</p> <p>17 exposure to talc and ovarian cancer."</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A Disagree.</p> <p>21 MS. THOMPSON:</p> <p>22 Q You disagree.</p> <p>23 58, "In studies where the exposure is</p> <p>24 simple (e.g., never versus ever use), recall bias</p>	<p>1 Object to the form.</p> <p>2 A I agree on that.</p> <p>3 MS. THOMPSON:</p> <p>4 Q 61, "The gold standard for translating</p> <p>5 epidemiologic case-controlled or cohort</p> <p>6 observational studies into a clinical meaningful</p> <p>7 data relies on laboratory-derived experiments in</p> <p>8 vitro or in vivo."</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A I disagree with that.</p> <p>12 MS. THOMPSON:</p> <p>13 Q On what basis?</p> <p>14 A The -- it depends upon the</p> <p>15 epidemiologic date that that we're talking about.</p> <p>16 Q In other words, if the epidemiologic</p> <p>17 data isn't strong enough, in your opinion, then</p> <p>18 doing in vitro or in vivo studies don't provide</p> <p>19 clinically meaningful data? Is that --</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A It's actually -- it's actually the</p> <p>23 other way around. So I think if it's a weak</p> <p>24 association, then the laboratory data becomes</p>
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<p>1 is unlikely to be an important source of bias."</p> <p>2 Agree or disagree?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A No opinion.</p> <p>6 MS. THOMPSON:</p> <p>7 Q Is that an issue that you would be</p> <p>8 inclined to -- to ask an epidemiologist?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A I'd like to see the -- I'd like to see</p> <p>12 the study that it's based on.</p> <p>13 MS. THOMPSON:</p> <p>14 Q Okay. 59, "Available data are</p> <p>15 indicative of a causal effect." And again,</p> <p>16 referring to talc and ovarian cancer.</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A Disagree.</p> <p>20 MS. THOMPSON:</p> <p>21 Q 60, "The data supporting the</p> <p>22 association of talc to the development of ovarian</p> <p>23 cancer is completely inconclusive."</p> <p>24 MS. CURRY:</p>	<p>1 that much more important for biologic</p> <p>2 plausibility.</p> <p>3 If it has -- you know, if it's chimney</p> <p>4 sweeps or lung cancer with smoking, then that's</p> <p>5 clinically meaningful. Those effects are huge.</p> <p>6 That's what I'm -- I'm not associating this just</p> <p>7 with the talc statement. Is it a talc statement?</p> <p>8 MS. THOMPSON:</p> <p>9 Q Uh-huh. I just want to make -- just</p> <p>10 want to make sure that I understand the -- the</p> <p>11 reason for your disagreement. But if you feel</p> <p>12 like it's explained, I'm good.</p> <p>13 A And again, I -- it's sort of the broad</p> <p>14 view that if -- if the -- if the epidemiologic</p> <p>15 case control and cohort studies are so powerful</p> <p>16 with a huge effect, then the biologic experiments</p> <p>17 and lab become less important.</p> <p>18 The other way around, which is really</p> <p>19 what we're dealing with with talc where the</p> <p>20 epidemiologic data I think is not compelling, the</p> <p>21 biologic plausibility becomes more important.</p> <p>22 And it sort of gets back into the Bradford Hill.</p> <p>23 Q Okay. So it's sort of inversely</p> <p>24 proportional in terms of the --</p>

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<p>1 A In terms of value.</p> <p>2 Q -- the importance of it?</p> <p>3 A Yeah.</p> <p>4 Q Okay. Got it.</p> <p>5 62, "Mineral talc occurs naturally in a</p> <p>6 platy, flat form, but may also occur as</p> <p>7 asbestiform fibers, which describes its physical</p> <p>8 form and does not imply the presence of asbestos.</p> <p>9 The purer forms, approximately 90 percent mineral</p> <p>10 talc, are used for" -- oops -- "are used for</p> <p>11 cosmetic and hygiene products, including baby</p> <p>12 powders and feminine hygiene products."</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 MS. THOMPSON:</p> <p>16 Q Agree or disagree or no opinion?</p> <p>17 A No opinion.</p> <p>18 Q That's it. I'll think of some new</p> <p>19 questions.</p> <p>20 A I feel like I just took my boards.</p> <p>21 Q Dr. Birrer, how do you define a</p> <p>22 carcinogen?</p> <p>23 A That's an agent or substance which</p> <p>24 causes or induces cancer.</p>	<p>1 Q Are you familiar with the term -- and I</p> <p>2 believe this is more in the toxicological</p> <p>3 literature -- of a complete carcinogen?</p> <p>4 A I would --</p> <p>5 Q Does that have a meaning to you?</p> <p>6 A Yeah. I've seen that described.</p> <p>7 Frankly, I can only -- I can only sort of guess</p> <p>8 what they mean by that. My guess is a complete</p> <p>9 carcinogen, putting out there for the discussion</p> <p>10 between you and me is what I'm describing as the</p> <p>11 classic initiation molecule.</p> <p>12 Q IARC describes -- do I have it? Would</p> <p>13 you look at Exhibit 6, which is the IARC? I just</p> <p>14 wanted to look at their definition of</p> <p>15 carcinogenesis and see whether you would agree</p> <p>16 with it or not.</p> <p>17 A Is it in the preamble?</p> <p>18 Q It's in the preamble. And if I can't</p> <p>19 find it, we may come back to that later.</p> <p>20 Because I can't remember where it is.</p> <p>21 Let's come back to that.</p> <p>22 A It's a big preamble.</p> <p>23 Q Lots of methodology.</p> <p>24 Are you familiar with the Hanahan paper</p>
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<p>1 Q Do you include effect on the promotion</p> <p>2 and progression of cancer as well in a -- when</p> <p>3 you're considering carcinogenicity?</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 A So historically -- and there's been a</p> <p>7 lot of work on this for decades -- carcinogens</p> <p>8 have been -- usually been associated with</p> <p>9 initiation. So this is a substance -- just to</p> <p>10 you an example. Paint it on to a mouse skin, and</p> <p>11 you develop tumors above -- statistically</p> <p>12 significantly above background.</p> <p>13 Tumor promoters don't do that. But</p> <p>14 when you combine the tumor promoter with the</p> <p>15 carcinogen, instead of getting the 10 tumors, now</p> <p>16 you get a hundred. So promotion is a little bit</p> <p>17 different. That's the historic perspective.</p> <p>18 You know, we've come a long way since</p> <p>19 then, and I think it's gotten even more complex,</p> <p>20 that there are tumor promoters that work by</p> <p>21 transcriptional factors. So that's not genetic</p> <p>22 changes in the tumor, in the cells. Carcinogens</p> <p>23 usually work that way, where you're getting a</p> <p>24 permanent genetic change.</p>	<p>1 from 2011 "Hallmarks of Cancer"?</p> <p>2 A It's a global sort of review. Yes.</p> <p>3 Q A big review --</p> <p>4 A Big.</p> <p>5 Q -- article?</p> <p>6 A Is it --</p> <p>7 Q Do you know -- do you know Dr. Hanahan</p> <p>8 or know of Dr. Hanahan?</p> <p>9 A I know of him.</p> <p>10 Q And it's Hanahan and Weinberg?</p> <p>11 A Weinberg, yeah. Yeah.</p> <p>12 Q Let me go ahead and mark that.</p> <p>13 A Okay.</p> <p>14 (DEPOSITION EXHIBIT NUMBER 10</p> <p>15 WAS MARKED FOR IDENTIFICATION.)</p> <p>16 MS. THOMPSON:</p> <p>17 Make sure those don't have my markings</p> <p>18 on it.</p> <p>19 A It would be easier for me if the</p> <p>20 markings were there.</p> <p>21 MS. THOMPSON:</p> <p>22 Q Exhibit 10. And you agree that this</p> <p>23 article describes the hallmarks of cancer in a</p> <p>24 general sense; right?</p>

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<p style="text-align: right;">Page 150</p> <p>1 A Correct.</p> <p>2 Q And it's a review article in Cell. Are</p> <p>3 you familiar with that journal?</p> <p>4 A I am.</p> <p>5 Q Have you published in that journal?</p> <p>6 Probably.</p> <p>7 A I wished I had published more in that</p> <p>8 journal. Yeah.</p> <p>9 Q And it's -- the title of the article is</p> <p>10 "The Hallmarks of Cancer: The Next Generation."</p> <p>11 But in the top right hand, it says, "Leading edge</p> <p>12 review." So that would be a review article for a</p> <p>13 general audience. Would you agree?</p> <p>14 A Yes. General audience of scientists,</p> <p>15 yeah. Because it's pretty sophisticated.</p> <p>16 Q Agree.</p> <p>17 And it describes the hallmarks of</p> <p>18 cancer generally. These do not specifically</p> <p>19 apply to ovarian cancer in -- in the</p> <p>20 introduction. I'm starting on the third</p> <p>21 sentence. "They include sustaining proliferative</p> <p>22 signaling, evading growth suppressors, resisting</p> <p>23 cell death, enabling replicative" --</p> <p>24 A Third line of -- you're in the abstract</p>	<p style="text-align: right;">Page 152</p> <p>1 Characteristics."</p> <p>2 And it says, the first sentence, "An</p> <p>3 increasing body of research suggests that two</p> <p>4 additional hallmarks of cancer are involved in</p> <p>5 the pathogenesis of some and perhaps all</p> <p>6 cancers."</p> <p>7 I'm gonna skip down to the -- to the</p> <p>8 last sentence in that description.</p> <p>9 "Inflammation" --</p> <p>10 A You're in the figure legend?</p> <p>11 Q In the figure legend.</p> <p>12 "Inflammation by innate immune cells</p> <p>13 designed to fight infections and heal wounds can</p> <p>14 instead result in their inadvertent support of</p> <p>15 multiple hallmark capabilities, thereby</p> <p>16 manifesting the now widely appreciated tumor</p> <p>17 promoting consequences of inflammatory</p> <p>18 responses."</p> <p>19 Would you agree with that statement, in</p> <p>20 a general sense?</p> <p>21 A Yes.</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A Sorry.</p>
<p style="text-align: right;">Page 151</p> <p>1 or in the introduction?</p> <p>2 Q I'm in the -- sorry. I'm in the</p> <p>3 abstract.</p> <p>4 A Okay.</p> <p>5 Q It sort of seemed more like an</p> <p>6 introduction than an abstract to me. So starting</p> <p>7 again. Talking about the hallmarks described in</p> <p>8 this paper, "They include sustaining</p> <p>9 proliferative signalling, evading growth</p> <p>10 suppressors, resisting cell death, enabling</p> <p>11 replicative immortality, enduing angiogenesis,</p> <p>12 and activating invasion and metastasis.</p> <p>13 "Underlining these hallmarks are genome</p> <p>14 instability which generates the genetic diversity</p> <p>15 that expedites their acquisition and</p> <p>16 inflammation, which fosters multiple hallmark</p> <p>17 functions."</p> <p>18 Would you agree with that statement</p> <p>19 from this article?</p> <p>20 A I think as a general statement, yes.</p> <p>21 Q And the article, as you described, is</p> <p>22 quite technical and -- and goes on for a while.</p> <p>23 I'm looking at the Figure 3 on page 658. And the</p> <p>24 heading is "Emerging Hallmarks and Enabling</p>	<p style="text-align: right;">Page 153</p> <p>1 MS. THOMPSON:</p> <p>2 Q Are you familiar with Dr. Balkwill?</p> <p>3 A We're done with this?</p> <p>4 Q We're done with that.</p> <p>5 A Fran? Fran Balkwill? Yes.</p> <p>6 Q And I believe you published with</p> <p>7 Dr. Balkwill?</p> <p>8 A I believe we're on two. I can't</p> <p>9 remember.</p> <p>10 Q And she is a well-renowned cancer</p> <p>11 biologist. Would you agree?</p> <p>12 A I would agree.</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 (DEPOSITION EXHIBIT NUMBER 11</p> <p>16 WAS MARKED FOR IDENTIFICATION.)</p> <p>17 MS. THOMPSON:</p> <p>18 Q I'm gonna mark as Exhibit 11 an article</p> <p>19 written by Dr. Balkwill.</p> <p>20 Have you seen this article, Dr. Birrer?</p> <p>21 A I'm actually not familiar with this.</p> <p>22 But I know Fran's work pretty well.</p> <p>23 Q Okay. Well, let's just --</p> <p>24 A Yeah.</p>

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<p>1 Q -- look through it. And this is also a</p> <p>2 review article.</p> <p>3 A Uh-huh.</p> <p>4 Q And -- and this article is in -- is in</p> <p>5 The Lancet. Correct?</p> <p>6 A Correct.</p> <p>7 Q And is -- we've already mentioned that</p> <p>8 Dr. Balkwill is well regarded.</p> <p>9 Is The Lancet a well-regarded journal?</p> <p>10 A Yes.</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 MS. THOMPSON:</p> <p>14 Q Is it one of the most respected</p> <p>15 journals, would you say?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A It's not as good as Cell.</p> <p>19 MS. THOMPSON:</p> <p>20 Q Oh. I won't tell them you said that.</p> <p>21 But, generally -- generally speaking --</p> <p>22 A Yes.</p> <p>23 Q -- physicians and scientists would</p> <p>24 recognize The Lancet?</p>	<p>1 progression, and immunosuppression than they are</p> <p>2 to mount an effective host antitumor response.</p> <p>3 Moreover cancer suscep- -- susceptibility and</p> <p>4 severity may be associated with functional</p> <p>5 polymorphisms of inflammatory cytokine genes, and</p> <p>6 deletion or inhibition of inflammatory cytokines,</p> <p>7 inhibits development of experimental cancer.</p> <p>8 "If genetic damage is the 'match that</p> <p>9 lights the fire' of cancer, some types of</p> <p>10 inflammation may provide the 'fuel that feeds the</p> <p>11 flames.'"</p> <p>12 That was a long passage, but do you</p> <p>13 generally agree with the statement by</p> <p>14 Dr. Balkwill?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A I do.</p> <p>18 MS. THOMPSON:</p> <p>19 Q And then look down on that same page to</p> <p>20 panel 1.</p> <p>21 A Uh-huh.</p> <p>22 Q And the title of that panel, for lack</p> <p>23 of better word, is "Some Associations Between</p> <p>24 Inflammation and Cancer Risk." Right?</p>
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<p>1 A It's well read -- it's well read and</p> <p>2 it's -- it has a substantial impact factor.</p> <p>3 Q And we don't know in this situation</p> <p>4 whether Dr. Balkwill -- do you know</p> <p>5 Dr. Mantovani, the second author on this paper?</p> <p>6 A No. I don't recognize him.</p> <p>7 Q We don't know whether this article was</p> <p>8 invited or submitted, but, regardless, certainly</p> <p>9 the readers of Lancet would look to Dr. Balkwill</p> <p>10 as being an expert to discuss inflammation in</p> <p>11 cancer; correct?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A Correct.</p> <p>15 MS. THOMPSON:</p> <p>16 Q So reading in -- in the abstract, which</p> <p>17 looks like an introduction to me again, but</p> <p>18 reading the abstract, "This article reviews" --</p> <p>19 second line -- "This article reviews the links</p> <p>20 between cancer and inflammation and discusses the</p> <p>21 implications of these links for cancer prevention</p> <p>22 and treatment. We suggest that the inflammatory</p> <p>23 cells and cytokines found in tumors are more</p> <p>24 likely to contribute to tumor growth,</p>	<p>1 A 901. Got it.</p> <p>2 Q And under "Malignancy," it lists</p> <p>3 various types of cancer in which there's</p> <p>4 association between inflammation and cancer risk.</p> <p>5 Correct?</p> <p>6 A Correct.</p> <p>7 Q And one of them -- one of them is</p> <p>8 ovarian; right?</p> <p>9 A I see it.</p> <p>10 Q And in the -- under the inflammatory</p> <p>11 stimulus/condition, it lists pelvic inflammatory</p> <p>12 disease, talc, tissue remodeling.</p> <p>13 Do you agree that Dr. Balkwill, at</p> <p>14 least in 2001, believed that talc was an</p> <p>15 inflammatory stimulus and condition for the</p> <p>16 association with ovarian cancer?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A Yeah. So, again, this is a -- a bit of</p> <p>20 a recurring theme in the sense that I don't know</p> <p>21 if Fran -- I haven't talked to her about this</p> <p>22 review. I don't know if Fran believed that and</p> <p>23 got it wrong or, more likely, this is a review</p> <p>24 article. So you include everything, even though</p>

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<p style="text-align: right;">Page 158</p> <p>1 she may not feel really strongly about that. So</p> <p>2 it's a little hard to tell.</p> <p>3 MS. THOMPSON:</p> <p>4 Q But you would agree that both -- both</p> <p>5 Dr. Balkwill and The Lancet would not include</p> <p>6 something in a review article for which there was</p> <p>7 no evidence?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Again, it depends on how they're</p> <p>11 proposing it; that there has been -- there has --</p> <p>12 there have been reports associating PID, talc --</p> <p>13 I don't know what tissue remodeling is, although</p> <p>14 that is probably the most reasonable -- but PID</p> <p>15 and talc as associated with a risk for ovarian</p> <p>16 cancer. That's a true statement. I don't -- and</p> <p>17 the reason we're here today is because I reviewed</p> <p>18 that literature and I don't believe the</p> <p>19 conclusion.</p> <p>20 But you could put it into review.</p> <p>21 That's -- that's the nature of a review article.</p> <p>22 We all put things in that we feel the reader</p> <p>23 needs to see to get a full understanding of</p> <p>24 science, but we don't necessarily -- we're not</p>	<p style="text-align: right;">Page 160</p> <p>1 them to say, okay, this has been studied</p> <p>2 epidemiologically and in other situations. So I</p> <p>3 think -- I think that's what you're grappling</p> <p>4 with. It's a review article. So these things</p> <p>5 show up.</p> <p>6 Q Okay. So -- so there are two</p> <p>7 possibilities --</p> <p>8 A Uh-huh.</p> <p>9 Q -- it sounds like. Either Dr. Balkwill</p> <p>10 got it wrong --</p> <p>11 A Uh-huh.</p> <p>12 Q -- or because this was a review</p> <p>13 article, she was reporting evidence that was in</p> <p>14 the literature that she felt that readers of this</p> <p>15 article should be aware of.</p> <p>16 A Correct. Don't tell her I said the</p> <p>17 former.</p> <p>18 MS. CURRY:</p> <p>19 Object to the form of the question.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Okay. I -- I -- I will do that for</p> <p>22 you, Dr. Birrer.</p> <p>23 A Uh-huh.</p> <p>24 Q And -- and this paper is not recent,</p>
<p style="text-align: right;">Page 159</p> <p>1 convinced.</p> <p>2 MS. THOMPSON:</p> <p>3 Q Well, but -- but back to my question,</p> <p>4 which I think was Dr. Balkwill and The Lancet</p> <p>5 would not have put this in with no evidence.</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A I don't agree with that.</p> <p>9 MS. THOMPSON:</p> <p>10 Q You think they would put something in</p> <p>11 that they did not believe there was any evidence</p> <p>12 to support?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A Again, it depends on how you define</p> <p>16 that. So when you say "no evidence," you mean no</p> <p>17 epidemiologic studies that have ever shown an</p> <p>18 association. We know that's not true. There</p> <p>19 have been some. So there is some evidence. It's</p> <p>20 the totality of the evidence that I don't</p> <p>21 believe.</p> <p>22 MS. THOMPSON:</p> <p>23 Q Okay.</p> <p>24 A But it would not be unreasonable for</p>	<p style="text-align: right;">Page 161</p> <p>1 you will agree?</p> <p>2 A 2010?</p> <p>3 Q 2001.</p> <p>4 A 2001. Uh-huh. Yeah. Okay.</p> <p>5 Q Are you aware of anything that</p> <p>6 Johnson & Johnson did in 2001 to address this</p> <p>7 idea of Dr. Balkwill and others, including</p> <p>8 Dr. Ness, that talc may be causing ovarian cancer</p> <p>9 through an inflammatory process?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A In 2000 -- in 2001?</p> <p>13 MS. THOMPSON:</p> <p>14 Q Right.</p> <p>15 Did Johnson & Johnson respond to what</p> <p>16 at least is reported as being in the literature</p> <p>17 in Lancet?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A I'm not aware of that.</p> <p>21 MS. THOMPSON:</p> <p>22 Q I'm gonna mark as Exhibit 13 --</p> <p>23 MS. EVERETT:</p> <p>24 12.</p>

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<p style="text-align: right;">Page 162</p> <p>1 MS. THOMPSON: 2 Q Oh, there it is. 3 (DEPOSITION EXHIBIT NUMBER 12 4 WAS MARKED FOR IDENTIFICATION.) 5 MS. THOMPSON: 6 Q Exhibit 12 is going to be another 7 article -- another review article by Dr. Reuter 8 and authors. Oh, we need to -- sorry. Make sure 9 that's not my copy. 10 A This is mine? 11 Q That's yours, yeah. 12 Are you familiar with the journal of 13 Free Radical Biology in Medicine? 14 A I am familiar. Not something I publish 15 in much. 16 Q And probably doesn't have quite the 17 reputation of The Lancet or Cell? 18 A I don't think so. 19 Q But regardless, it's peer-reviewed. 20 A Uh-huh. 21 Q Are you familiar with any of these 22 authors? 23 A Not firsthand. Aggarwal I may have 24 heard about, but not, firsthand, no.</p>	<p style="text-align: right;">Page 164</p> <p>1 A Where are you now? 2 Q I'm turning to page 2, 1604 in the 3 introduction section. 4 A Uh-huh. 5 Q The second paragraph reads "Under a 6 sustained environmental stress, ROS -- R-O-S -- 7 are produced over a long time, and thus 8 significant damage may occur to cell structure 9 and functions and may induce somatic mutations 10 and neoplastic transformation. 11 "Indeed, cancer initiation and 12 progression have been linked to oxidative stress 13 by increasing DNA mutations or inducing DNA 14 damage, genome instability, and cell 15 proliferation." 16 Would you agree with that sentence in a 17 general sense? 18 MS. CURRY: 19 Object to the form. 20 A I'm just looking at the references. 21 MS. THOMPSON: 22 Q And take a moment if you need to do 23 that. 24 A Sure.</p>
<p style="text-align: right;">Page 163</p> <p>1 Q And reading -- and the title of this 2 review article is "Oxidative stress, 3 inflammation, and cancer. How are they linked?" 4 Right? 5 A Correct. 6 Q Reading in the abstract, the last 7 couple of sentences starting with "How oxidative 8 stress activates inflammatory pathways leading to 9 a transformation of a normal cell to tumor cell, 10 tumor cell survival, proliferation, 11 chemoresistance, radioresistance, invasion, 12 angiogenesis, and stem cell survival is the focus 13 of this review. Overall, observations to date 14 suggest that oxidative stress, chronic 15 inflammation, and cancer are closely linked." 16 Would you agree with that statement? 17 MS. CURRY: 18 Object to the form. 19 A Yes. 20 MS. THOMPSON: 21 Q In a general sense, in a review 22 article? 23 A Correct. 24 Q And --</p>	<p style="text-align: right;">Page 165</p> <p>1 I think as a general statement, I 2 wouldn't -- I would not disagree with that. I 3 think that's -- yeah. 4 Q Sorry. 5 A Go ahead. 6 Q And this article was published in 2010; 7 correct? 8 A Correct. 9 Q And looking at Table 2, a partial list 10 of cancers that have been linked to reactive 11 oxygen species, and under that list is ovarian 12 cancer. 13 Would you agree that in 2010 ovarian 14 cancer had been linked to reactive oxygen 15 species? 16 MS. CURRY: 17 Object to the form. 18 A Yeah. This was a little more 19 complicated in the sense I'm not sure why every 20 case was not listed because reactive oxygen 21 species are present in essentially every cell in 22 the body. So it's a -- it's an odd table in that 23 it's a subset and then -- it's sort of implying 24 reactive oxygen species are not important in</p>

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<p style="text-align: right;">Page 166</p> <p>1 other cancers.</p> <p>2 And, then, too, what they reference is</p> <p>3 51, which is a really odd reference. "Loss of</p> <p>4 Mkp3 mediated by oxidative stress enhances tumor</p> <p>5 genicity and chemoresistance of ovarian cancer</p> <p>6 cells."</p> <p>7 Hardly a paper -- I mean, I'm</p> <p>8 extrapolating the title. Hardly a paper that</p> <p>9 would say that reactive oxygen species is</p> <p>10 critical to the development of ovarian cancer.</p> <p>11 That's chemoresistance. That's -- that's at the</p> <p>12 end of natural history, so...</p> <p>13 MS. THOMPSON:</p> <p>14 Q But at least the authors in this</p> <p>15 peer-reviewed review article thought appropriate</p> <p>16 to list ovarian cancer under one of the cancers</p> <p>17 that have been linked to reactive oxygen species;</p> <p>18 right?</p> <p>19 A It's there.</p> <p>20 (DEPOSITION EXHIBIT NUMBER 13</p> <p>21 WAS MARKED FOR IDENTIFICATION.)</p> <p>22 MS. THOMPSON:</p> <p>23 Q I'm marking as Exhibit 13 another</p> <p>24 review article from Lancet. This one, a little</p>	<p style="text-align: right;">Page 168</p> <p>1 Object to the form.</p> <p>2 A Oza and Vergote are -- Vergote is a</p> <p>3 surgeon and very much clinical. I don't think he</p> <p>4 does any work in the lab. Oza is developmental</p> <p>5 therapeutics clinical. Charlie is the scientist</p> <p>6 here.</p> <p>7 MS. THOMPSON:</p> <p>8 Q Okay. And I think --</p> <p>9 A Yeah.</p> <p>10 Q -- at least with this review article,</p> <p>11 it was meant to address --</p> <p>12 A Everything.</p> <p>13 Q -- all -- all aspects --</p> <p>14 A Right.</p> <p>15 Q -- from my reading of it.</p> <p>16 A And I think Stephanie works for Amit, I</p> <p>17 think.</p> <p>18 Q So these are well-regarded --</p> <p>19 A Uh-huh.</p> <p>20 Q -- scientists and experts in ovarian</p> <p>21 cancer. You would agree?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A Yes.</p>
<p style="text-align: right;">Page 167</p> <p>1 more current.</p> <p>2 Have you seen this article, Dr. Birrer?</p> <p>3 A I know the -- I know the authors, but I</p> <p>4 haven't actually --</p> <p>5 Q Oh. Did I give you a highlighted --</p> <p>6 A I -- I don't think so.</p> <p>7 Q Okay.</p> <p>8 A It would be helpful if it was</p> <p>9 highlighted.</p> <p>10 Q It would be helpful to me also.</p> <p>11 That's okay.</p> <p>12 And, in fact, these -- I think three of</p> <p>13 the four authors you have published with. Does</p> <p>14 that sound right?</p> <p>15 A Ignace, Charlie, Amit, I know all of</p> <p>16 them. I don't know Stephanie.</p> <p>17 Q I think that was the one that I did not</p> <p>18 see on -- on your CV as one of your coauthors.</p> <p>19 And this review article -- and you</p> <p>20 would assume that -- well, we don't have to</p> <p>21 assume -- are Dr. Gourley, Dr. Vergote and</p> <p>22 Dr. Oza considered experts in the field of</p> <p>23 epithelial ovarian cancer?</p> <p>24 MS. CURRY:</p>	<p style="text-align: right;">Page 169</p> <p>1 MS. THOMPSON:</p> <p>2 Q And this is a review article, as we</p> <p>3 said, just published in Lancet within -- March</p> <p>4 23rd, so within the last week.</p> <p>5 Have you seen this article?</p> <p>6 A This one?</p> <p>7 Q Yes.</p> <p>8 A No. Just the last week.</p> <p>9 Q Let's look in the first section,</p> <p>10 Epidemiology and Risk Factors. And the last</p> <p>11 sentence, "Risk factors for EOC include the</p> <p>12 number of lifetime of ovulations (absence of</p> <p>13 pregnancy), early age of menarche and late age at</p> <p>14 menopause, family history of EOC, smoking, benign</p> <p>15 gynecological conditions, including</p> <p>16 endometriosis -- endometriosis, polycystic ovary</p> <p>17 disease and pelvic inflammatory disease, and</p> <p>18 potentially use of talcum powder."</p> <p>19 Would you agree that at least the</p> <p>20 authors thought that the use of talcum powder is</p> <p>21 potentially a risk factor for EOC?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A And, again, this is a review. So I</p>

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<p style="text-align: right;">Page 170</p> <p>1 think they're trying to be inclusive. And I</p> <p>2 don't actually know that any of them believe</p> <p>3 that.</p> <p>4 MS. THOMPSON:</p> <p>5 Q So would -- would they -- would they</p> <p>6 have -- would it be the two options again, either</p> <p>7 they're wrong --</p> <p>8 A (Nods affirmatively.)</p> <p>9 Q -- or that they're just reporting on</p> <p>10 what the literature states?</p> <p>11 A (Nods affirmatively.)</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A Yeah. I think it extends beyond</p> <p>15 talcum, too, to be honest with you. I don't -- I</p> <p>16 don't consider smoking to be a strong risk for</p> <p>17 ovarian cancer. And PID, I don't either.</p> <p>18 So -- and I don't know of many of my --</p> <p>19 I mean, we don't -- we don't want our patients</p> <p>20 smoking. But I don't know of many of the</p> <p>21 gynecologic oncologists I work with who -- that's</p> <p>22 on their -- that's on their risk list.</p> <p>23 MS. THOMPSON:</p> <p>24 Q Even for mucinous?</p>	<p style="text-align: right;">Page 172</p> <p>1 Q So the authors, if they were reporting</p> <p>2 on the potential risk of talcum powder use in</p> <p>3 ovarian cancer chose to cite Penninkilampi as a</p> <p>4 source -- as the source for that information;</p> <p>5 correct?</p> <p>6 A They reference it.</p> <p>7 Q And you would assume they would choose</p> <p>8 the most authoritative article that was available</p> <p>9 in the literature?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 MS. THOMPSON:</p> <p>13 Q Wouldn't you?</p> <p>14 A I would not assume that.</p> <p>15 Q You would assume they'd pick something</p> <p>16 that wasn't as authoritative? There's something</p> <p>17 else they could have picked?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A They may have -- they may have picked</p> <p>21 that because it was one of the more recent</p> <p>22 meta-analyses, and so it was convenient. And</p> <p>23 it's flawed. We can go over if you'd like.</p> <p>24 MS. THOMPSON:</p>
<p style="text-align: right;">Page 171</p> <p>1 A Well, now you're gonna get complicated</p> <p>2 on me because, you know, there are people that</p> <p>3 don't think -- there are mucinous tumors of the</p> <p>4 ovary. Bob Kirkman is one of them, and that is</p> <p>5 all GI.</p> <p>6 So I think -- I don't think it's all</p> <p>7 that relevant because it's such a rare tumor.</p> <p>8 Q And the citation for the reference</p> <p>9 that --</p> <p>10 A 8?</p> <p>11 Q -- a risk factor potentially would --</p> <p>12 could be the use of talcum powder is the</p> <p>13 Penninkilampi meta-analysis; right?</p> <p>14 A That's referenced in 8, yes.</p> <p>15 Q So at least the authors, the reviewers,</p> <p>16 the editors of the journal felt that the most</p> <p>17 authoritative source would be that Penninkilampi</p> <p>18 meta-analysis. Would you agree?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A Say that again. I'm sorry.</p> <p>22 MS. THOMPSON:</p> <p>23 Q Yeah.</p> <p>24 A I could read it.</p>	<p style="text-align: right;">Page 173</p> <p>1 Q Well, I'm just saying these authors</p> <p>2 picked that to -- to support the statement in</p> <p>3 their review article in The Lancet that the use</p> <p>4 of talcum powder is potentially a risk factor for</p> <p>5 ovarian cancer.</p> <p>6 A Well, I would agree that they picked</p> <p>7 that reference. I disagree that that's because</p> <p>8 they thought it was the most authoritative</p> <p>9 article. It is one of the more recent, and, so,</p> <p>10 therefore, a lot of the other papers would be</p> <p>11 included in it. So it's a convenient place to</p> <p>12 steer a reader.</p> <p>13 Q Do you think they'd pick it if they</p> <p>14 thought it was flawed?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A Probably if -- if it was seriously</p> <p>18 flawed, I don't think they would have picked it.</p> <p>19 Yeah.</p> <p>20 MS. THOMPSON:</p> <p>21 Q And would you agree, also, that the</p> <p>22 reviewers would not have included an article that</p> <p>23 the reviewers felt was seriously flawed?</p> <p>24 MS. CURRY:</p>

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<p style="text-align: right;">Page 174</p> <p>1 Object to the form.</p> <p>2 A Again, it's a little bit -- having been</p> <p>3 involved in these processes, to be perfectly</p> <p>4 frank, you get a review article with a review of</p> <p>5 147 references, you're not gonna go through them</p> <p>6 all. So I don't know I can say with any</p> <p>7 authority that the reviewers looked at this and</p> <p>8 said, gee, they picked the one talc paper that is</p> <p>9 really spectacular.</p> <p>10 MS. THOMPSON:</p> <p>11 Q Okay. So there were -- but there --</p> <p>12 there were no --</p> <p>13 A The review, and -- and it's true for</p> <p>14 the editor too.</p> <p>15 Q Okay. So at least there were no red</p> <p>16 flags in front of the reviewers and the editor</p> <p>17 when they saw the Penninkilampi article cited for</p> <p>18 that reference?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A I --</p> <p>22 MS. THOMPSON:</p> <p>23 Q That would cause them to --</p> <p>24 A I don't know they noticed it.</p>	<p style="text-align: right;">Page 176</p> <p>1 lunch?</p> <p>2 MS. CURRY:</p> <p>3 We actually did order in lunch. I'm</p> <p>4 not sure if we -- if you want to take a quick</p> <p>5 break, I can check on the estimated time of</p> <p>6 arrival.</p> <p>7 MS. THOMPSON:</p> <p>8 Sure. Or we can just keep going until</p> <p>9 we get word. Whatever --</p> <p>10 A Or we could just finish.</p> <p>11 MR. MIZGALA:</p> <p>12 I second that.</p> <p>13 MS. GARBER:</p> <p>14 You guys keep going. I'll check.</p> <p>15 MS. THOMPSON:</p> <p>16 Are you telling me you're not having</p> <p>17 fun? I think he liked the test.</p> <p>18 THE WITNESS:</p> <p>19 Yeah. It would have been nice to have</p> <p>20 the little box -- the little circles you could</p> <p>21 fill in. You know.</p> <p>22 MS. THOMPSON:</p> <p>23 And then I could just put it in the</p> <p>24 computer.</p>
<p style="text-align: right;">Page 175</p> <p>1 Q Okay. But the editors selected that</p> <p>2 article; correct?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 MS. THOMPSON:</p> <p>6 Q For whatever reason?</p> <p>7 A The --</p> <p>8 Q The authors.</p> <p>9 A The authors selected it.</p> <p>10 Q Sorry.</p> <p>11 A Not -- not the editors. Correct.</p> <p>12 Q Thank you. I meant to say authors.</p> <p>13 A And, again, I would just emphasize it</p> <p>14 says "potentially use of talcum powder."</p> <p>15 Q That's right.</p> <p>16 A Okay.</p> <p>17 Q And at least in this statement, the</p> <p>18 reference to talcum powder as potentially a risk</p> <p>19 factor did not separate out the subtypes. It's</p> <p>20 referring to EOC; correct?</p> <p>21 A I -- that's the way I would read it,</p> <p>22 right.</p> <p>23 MS. THOMPSON:</p> <p>24 Dawn, what are you thinking about</p>	<p style="text-align: right;">Page 177</p> <p>1 THE WITNESS:</p> <p>2 No mumbling? Sorry.</p> <p>3 MS. CURRY:</p> <p>4 Okay. So the lunch, I was just told,</p> <p>5 is actually here. So it's up to you when you're</p> <p>6 in a good breaking point.</p> <p>7 MS. THOMPSON:</p> <p>8 Dr. Birrer, do you want to take a break</p> <p>9 for lunch or do you want to go another 15 or 20</p> <p>10 minutes?</p> <p>11 THE WITNESS:</p> <p>12 Going would be fine.</p> <p>13 MS. THOMPSON:</p> <p>14 Q Okay.</p> <p>15 A Yeah.</p> <p>16 Q Let's -- let's look at the IARC 93, the</p> <p>17 one that --</p> <p>18 A Uh-huh.</p> <p>19 Q -- addresses the nonasbestiform talc.</p> <p>20 And turning to page 277 in the exposure data</p> <p>21 introduction --</p> <p>22 A Uh-huh. Do you want to use mine?</p> <p>23 Q Let's have a blank one to follow along.</p> <p>24 Does this section define the</p>

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<p style="text-align: right;">Page 178</p> <p>1 nonasbestiform talc?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 MS. THOMPSON:</p> <p>5 Q Oh, there it is. And let's just read</p> <p>6 along in that third paragraph.</p> <p>7 A Okay.</p> <p>8 Q "Asbestiform talc fibers are very long</p> <p>9 and thin and occur in parallel bundles that are</p> <p>10 easily separated from one another by hand</p> <p>11 pressure." And asbestos -- no. Just strike</p> <p>12 that.</p> <p>13 You're -- you're not an expert in the</p> <p>14 different types of asbestos or talc in its</p> <p>15 different --</p> <p>16 A I'm learning --</p> <p>17 Q Are you?</p> <p>18 A I'm learning a lot.</p> <p>19 Q I -- well, I don't want to ask those</p> <p>20 questions to you later because then you'll be an</p> <p>21 expert.</p> <p>22 Let's -- let's go to the conclusions of</p> <p>23 IARC. We've already established that IARC used a</p> <p>24 pretty extensive methodology in reaching their</p>	<p style="text-align: right;">Page 180</p> <p>1 was -- well, that there was limited evidence in</p> <p>2 humans for the carcinogenicity in peroneal use of</p> <p>3 talcum powder body product. Is that what IARC</p> <p>4 concluded?</p> <p>5 A That's in 6.1, the second one. Yes.</p> <p>6 Q Right.</p> <p>7 And there is limited evidence in</p> <p>8 experimental animals; right?</p> <p>9 A 6.2. Yes.</p> <p>10 Q And in the rationale, the authors</p> <p>11 state, third paragraph, "For peroneal use of</p> <p>12 talcum-based body power, many case-control</p> <p>13 studies of ovarian cancer found a modest but an</p> <p>14 unusually consistent excessive risk, although the</p> <p>15 impact of bias and potential confounding could</p> <p>16 not be ruled out."</p> <p>17 Is -- is that your understanding of the</p> <p>18 conclusions?</p> <p>19 A That's what they concluded.</p> <p>20 Q And --</p> <p>21 A We're done with IARC?</p> <p>22 Q We're done with IARC.</p> <p>23 And you also looked at the Health</p> <p>24 Canada Assessment; right?</p>
<p style="text-align: right;">Page 179</p> <p>1 conclusions; right?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 A Yes.</p> <p>5 MS. THOMPSON:</p> <p>6 Q And in your -- in your opinion, IARC</p> <p>7 got -- got it wrong; right?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A I think the net -- and I -- let me just</p> <p>11 summarize. I agree that they did a thorough sort</p> <p>12 of process here. In the end, what they</p> <p>13 concluded, I think, was -- was wrong. If I</p> <p>14 recall correctly, it's 2B.</p> <p>15 MS. THOMPSON:</p> <p>16 Q That's right.</p> <p>17 A Was the classification.</p> <p>18 Q But 2B does not mean that it's not</p> <p>19 carcinogenic, does it?</p> <p>20 A Means it's possible carcinogenic. I</p> <p>21 think that's by definition.</p> <p>22 Q Right.</p> <p>23 And -- and in this situation, the</p> <p>24 reason for the classification was that there</p>	<p style="text-align: right;">Page 181</p> <p>1 A Yes.</p> <p>2 Q And we agreed that the methodology that</p> <p>3 Health Canada applied for -- for their</p> <p>4 determination was also extensive; right?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A They were systematic and thorough. I</p> <p>8 think it was pretty complicated, yeah.</p> <p>9 MS. THOMPSON:</p> <p>10 Q And what's your understanding of the</p> <p>11 conclusions reached by the -- Health Canada?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Scientists.</p> <p>16 A Well, they concluded that there was a</p> <p>17 low risk of harm to the environment from talc.</p> <p>18 Q Is that what you came away with?</p> <p>19 A Well, it was in the third paragraph.</p> <p>20 So it was important to note that. But they did</p> <p>21 conclude that talc meets one of the criteria.</p> <p>22 That was Section 64. And so they concluded that</p> <p>23 it potentially presented a health risk to</p> <p>24 Canadians, if I got that right.</p>

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<p>1 Q And do you think it was just to</p> <p>2 Canadians?</p> <p>3 A Well, that's the way they quoted it.</p> <p>4 Q And --</p> <p>5 A In fact, the statement is "may</p> <p>6 constitute a danger in Canada to health" --</p> <p>7 "human health" -- "human life or health."</p> <p>8 Q And they also made the -- well, let's</p> <p>9 read beginning on page little -- little 3, i --</p> <p>10 iii?</p> <p>11 A I'm sorry. Where are you?</p> <p>12 Q Little -- little roman numeral 3.</p> <p>13 A Three? Yeah.</p> <p>14 Q Is your understanding that the -- that</p> <p>15 Health Canada found that the available data were</p> <p>16 indicative of a causal effect?</p> <p>17 A Where are you reading?</p> <p>18 Q I was just asking you what your</p> <p>19 understanding was.</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A I'm not sure that they actually found</p> <p>23 causal effects.</p> <p>24 MS. THOMPSON:</p>	<p>1 Q -- executive summary.</p> <p>2 A Yeah. Uh-huh.</p> <p>3 Q "Given that there is potential for</p> <p>4 peroneal exposure to talc from the use of various</p> <p>5 self-care products, for example, body powder,</p> <p>6 baby powder, diaper and rash creams, gentle</p> <p>7 antiperspirants and deodorants, body wipes, bath</p> <p>8 bombs, a potential concern for human health has</p> <p>9 been identified."</p> <p>10 Correct?</p> <p>11 A I agree with that.</p> <p>12 Q And is it your opinion that Health</p> <p>13 Canada got it wrong also?</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 A So it's interesting. When I reviewed</p> <p>17 this was -- again, this is a very recent -- looks</p> <p>18 like December 2018 -- decision by Health Canada</p> <p>19 based upon a huge body of literature, which I had</p> <p>20 reviewed and come to a different conclusion.</p> <p>21 So there really was not very much new</p> <p>22 data to draw this conclusion. So, you know,</p> <p>23 again, I think very much like IARC, I think they</p> <p>24 got it wrong.</p>
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<p>1 Q Okay. Well, let's -- let's read</p> <p>2 beginning -- the paragraph with "The</p> <p>3 meta-analyses."</p> <p>4 A Where are you? Oh, the -- yeah.</p> <p>5 Q "The meta-analyses of the available</p> <p>6 human studies in the peer-reviewed literature" --</p> <p>7 A Yep.</p> <p>8 Q -- "indicate a statistically</p> <p>9 significant positive association between perineal</p> <p>10 exposure to talc and ovarian cancer. Further,</p> <p>11 available data are indicative of a causal</p> <p>12 effect."</p> <p>13 A Uh-huh.</p> <p>14 Q So they did --</p> <p>15 A (Nods affirmatively.)</p> <p>16 Q -- determine that it was indicative of</p> <p>17 a causal effect; right?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A That's what they said, yes. It's not</p> <p>21 referenced, but --</p> <p>22 MS. THOMPSON:</p> <p>23 Q Well, this is the --</p> <p>24 A Yeah.</p>	<p>1 MS. THOMPSON:</p> <p>2 Q And you don't think that this is a</p> <p>3 situation where scientists can look at the same</p> <p>4 data and -- and make different conclusions?</p> <p>5 A No.</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 MS. THOMPSON:</p> <p>9 Q Do you have any reason to believe that</p> <p>10 the scientists who worked on this project were</p> <p>11 unreasonable?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A Other than the fact they drew the wrong</p> <p>15 conclusion here, I know nothing else about them,</p> <p>16 so...</p> <p>17 MS. THOMPSON:</p> <p>18 Q You don't have any reason to believe</p> <p>19 they were incompetent?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A No.</p> <p>23 MS. THOMPSON:</p> <p>24 Q Do you have any reason to believe that</p>

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<p>1 they weren't good scientists?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 A I don't really have a lot of knowledge</p> <p>5 of them. If I could actually find the list of</p> <p>6 individuals who made this decision -- I don't</p> <p>7 think it's published.</p> <p>8 MS. THOMPSON:</p> <p>9 Q And did you -- this was done under the</p> <p>10 auspices, I believe, of the Minister of Health.</p> <p>11 A Uh-huh.</p> <p>12 Q You don't know the Minister of Health</p> <p>13 in Canada, do you?</p> <p>14 A I don't.</p> <p>15 Q Or know that he would -- or she would</p> <p>16 not be competent?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A I have no direct evidence for that.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Do you take any issue with the weight</p> <p>22 of the evidence methodology that Health Canada</p> <p>23 applied?</p> <p>24 A No.</p>	<p>1 A In terms of peer review, scientific</p> <p>2 peer review?</p> <p>3 Q Correct.</p> <p>4 A I can't say that definitively.</p> <p>5 Q If you'll look at the -- and the copy</p> <p>6 that I'm looking at doesn't have page numbers, so</p> <p>7 that's why it's -- I'm --</p> <p>8 A Roughly.</p> <p>9 Q -- making it difficult.</p> <p>10 But if you look at the big bold</p> <p>11 introduction that comes right after the synopsis,</p> <p>12 it should be about the -- it may be the little</p> <p>13 numbers.</p> <p>14 A Introduction?</p> <p>15 Q Yeah.</p> <p>16 And the very bottom of that page, I'm</p> <p>17 reading "The human health portion of this</p> <p>18 assessment has undergone external peer review</p> <p>19 and/or consultation?"</p> <p>20 Doesn't -- does the assessment, at</p> <p>21 least, state that it underwent peer review and</p> <p>22 consultation?</p> <p>23 A It states that. I don't quite -- I</p> <p>24 don't honestly know what that means.</p>
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<p>1 Q Only that they came up with the wrong</p> <p>2 conclusion; right?</p> <p>3 A Correct.</p> <p>4 Q And this assessment, like IARC, was</p> <p>5 based on talc -- cosmetic-grade talc and not on</p> <p>6 potential impurities such as asbestos. Is that</p> <p>7 also your understanding?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A That is my understanding. So, you</p> <p>11 know, again, it's -- it's the same epi data. The</p> <p>12 epi data is focused on talcum powder. So that --</p> <p>13 that applies here, too.</p> <p>14 MS. THOMPSON:</p> <p>15 Q And is it your understanding that the</p> <p>16 human health portion of the Health Canada</p> <p>17 assessment went through a peer-review process?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 MS. THOMPSON:</p> <p>21 Q With external reviewers.</p> <p>22 A I didn't see that described.</p> <p>23 Q So you don't know one way or the other</p> <p>24 whether it went through a review process?</p>	<p>1 Q Okay.</p> <p>2 A And the public comment period, of</p> <p>3 course, is just a governmental response.</p> <p>4 Q Do you know if Johnson & Johnson has</p> <p>5 submitted comments to Health Canada?</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A Not that I know of.</p> <p>9 MS. THOMPSON:</p> <p>10 Q Have you submitted comments to Health</p> <p>11 Canada --</p> <p>12 A No.</p> <p>13 Q -- with your opinions?</p> <p>14 A No.</p> <p>15 Q Do you intend to submit any opinions to</p> <p>16 Health Canada?</p> <p>17 A I doubt it.</p> <p>18 Q You are -- are you aware that talc used</p> <p>19 as a dry powder lubricant on condoms was</p> <p>20 substituted with cornstarch in the 1990s?</p> <p>21 A I believe I am familiar with that.</p> <p>22 Q Do you know why?</p> <p>23 A No.</p> <p>24 Q Do you know that dusting diaphragms,</p>

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<p style="text-align: right;">Page 190</p> <p>1 the practice of dusting diaphragms with talcum 2 powder was abandoned approximately the same time? 3 MS. CURRY: 4 Object to the form. 5 A Yes. 6 MS. THOMPSON: 7 Q Do you know why? 8 A No. 9 Q Was it for concerns about inflammatory 10 and cancer effects? 11 MS. CURRY: 12 Object to the form. 13 A Could have been. I don't -- can't 14 quote that. 15 MS. THOMPSON: 16 Q Were you aware that FDA banned -- has 17 banned powder examination and surgical gloves? 18 A Yes. 19 Q Do you know why? 20 A That was based upon the concern about 21 the generation of fibrosis. 22 Q And other inflammatory processes in 23 the -- in the peritoneal cavity? 24 MS. CURRY:</p>	<p style="text-align: right;">Page 192</p> <p>1 Q Are you aware of the differences 2 between cornstarch and talc? 3 MS. CURRY: 4 Object to the form. 5 A In terms of biochemical and physical 6 differences? 7 MS. THOMPSON: 8 Q Sure. Let's start there. 9 A Yeah. I don't think I can list them 10 all. But certainly cornstarch is a biologic 11 agent, it's a carbohydrate, and talc is a 12 mineral. 13 We've already talked a little bit about 14 the size of particles in talcum powder and it's 15 exceedingly variable. So it's a little hard to 16 compare those two particles. But I would think 17 that starch would be more homogeneous and of a 18 different size. 19 And then, you know, biochemical 20 differences are substantial. I mean, this is a 21 carbohydrate, which can be broken down by certain 22 enzymes, has, you know, a firm structure to it. 23 Talc, as a mineral, forms suspensions. 24 It is not soluble. Starch is more soluble. So</p>
<p style="text-align: right;">Page 191</p> <p>1 Object to the form. 2 A I would define -- I would define that 3 as fibrosis, if not inflammatory. 4 MS. THOMPSON: 5 Q Do you consider granulomas an 6 inflammatory response? 7 A It's in the characterization of chronic 8 inflammation, yes. 9 Q Are adhesions an inflammatory response? 10 A Not necessarily. 11 Q And they would be an acute response 12 if -- if they were caused by an inflammatory 13 reaction? 14 MS. CURRY: 15 Object to the form. 16 A So adhesions are, you know, essentially 17 scar tissue and fibrosis. The etiology of it is 18 pretty broad. Some of it could be chronic 19 inflammation. Some of it could be acute 20 inflammation. And I would not even rule out the 21 possibility that general wound healing would give 22 rise to scar tissue. And that may not 23 necessarily fit the criteria of inflammation. 24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 193</p> <p>1 there's differences. 2 Q So, in general terms, cornstarch would 3 typically be absorbed or metabolized by the body? 4 MS. CURRY: 5 Object to the form. 6 MS. THOMPSON: 7 Q Would you agree? 8 A Absorbed or -- there's -- it would 9 certainly be more likely, I think, than a 10 mineral, yeah. 11 Q Whereas the mineral, once it's there, 12 is expected to remain there; correct? 13 MS. CURRY: 14 Object to the form. 15 A It's a little hard to tell because then 16 there are other mechanisms remove particulate 17 matters; right? So macrophages come along and 18 they phagocytize them. That macrophage then may 19 travel somewhere else and then essentially 20 deposit it in a way that the mineral -- the 21 mineral particle could be removed. So -- so it's 22 a little bit complex. 23 MS. THOMPSON: 24 Q Can inhaled talc particles appear in</p>

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<p>1 distant organs?</p> <p>2 A So there is some data, I believe, in</p> <p>3 animal studies that high concentrations of talc,</p> <p>4 either in the pleural cavity or in intratracheal</p> <p>5 injections can end up in what --</p> <p>6 And I think I put them in the expert</p> <p>7 report; for instance, the spleen.</p> <p>8 Q And ovaries? Can they occur in the</p> <p>9 ovaries?</p> <p>10 A So if you look at the literature -- you</p> <p>11 know, and I went through in pretty big detail --</p> <p>12 nobody's looked. So there's no reproductive</p> <p>13 organs in any of those studies. At least the</p> <p>14 ones that I have looked at. So I don't think we</p> <p>15 know, and I don't think we could assume that.</p> <p>16 Q Can talc fibers enter the peritoneal</p> <p>17 cavity?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A Again, we're back to this mineral</p> <p>21 structure, and I'm not going to be able to</p> <p>22 comment on that.</p> <p>23 MS. THOMPSON:</p> <p>24 Q And how about asbestos fibers?</p>	<p>1 know that.</p> <p>2 Q So you know -- you -- we know that</p> <p>3 asbestos fibers can reach the peritoneal cavity;</p> <p>4 correct?</p> <p>5 A Yes.</p> <p>6 Q And -- and let me just understand</p> <p>7 you -- what you're opining today is that we just</p> <p>8 don't know how they get there?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A I don't know. So -- so I think one of</p> <p>12 the hypotheses that -- after asbestos -- again,</p> <p>13 I'm not -- I wasn't asked to explore asbestos in</p> <p>14 great detail. This is more my medical training</p> <p>15 speaking.</p> <p>16 But as people inhaled asbestos, these</p> <p>17 particles would work their way out into the</p> <p>18 pleural cavity --</p> <p>19 MS. THOMPSON:</p> <p>20 Q So --</p> <p>21 A -- which is where they would do their</p> <p>22 badness. And then, there is a hypothesis</p> <p>23 connection between the pleural cavity and the</p> <p>24 peritoneal cavity.</p>
Page 195	Page 197
<p>1 A Well, asbestos exposure can, of course,</p> <p>2 give rise to mesothelioma and can give rise to</p> <p>3 peritoneal mesotheliomas. So it's got to get</p> <p>4 there from somewhere.</p> <p>5 Q Do you have an opinion as to whether</p> <p>6 asbestos fibers can get to the peritoneal cavity</p> <p>7 through peritoneal exposure and migration through</p> <p>8 the genital tract?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A I don't have any data on that.</p> <p>12 MS. THOMPSON:</p> <p>13 Q So you have no opinion.</p> <p>14 A I would say analogous with the</p> <p>15 migration data that there's not a lot of evidence</p> <p>16 things are migrating retrograde. So -- and I</p> <p>17 think -- although I don't think those experiments</p> <p>18 have been done with asbestos in mind -- and we</p> <p>19 know that asbestos can travel with high</p> <p>20 insulation [sic] -- you know, inhalation of</p> <p>21 asbestos can get in the pleural cavity. It gets</p> <p>22 there from somewhere. It's got to be inside the</p> <p>23 lung. It has to get out in the pleural cavity,</p> <p>24 and then again, the peritoneal cavity. So we</p>	<p>1 Q So direct penetration of the fiber</p> <p>2 through the pleura?</p> <p>3 A The diaphragm's are pretty secure</p> <p>4 structures, so it's a little bit -- I can't say,</p> <p>5 hey, here's the pathway. But that's the</p> <p>6 supposition.</p> <p>7 Q Okay.</p> <p>8 A Okay.</p> <p>9 Q Do you -- are you aware of any</p> <p>10 epidemiologic or other studies that have linked</p> <p>11 the use of perineal cornstarch with ovarian</p> <p>12 cancer?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A Perineal cornstarch with ovarian</p> <p>16 cancer?</p> <p>17 MS. THOMPSON:</p> <p>18 Q Correct. Let me phrase that</p> <p>19 differently just so it's clear.</p> <p>20 A Okay.</p> <p>21 Q Are you aware of any studies that link</p> <p>22 the perineal use of cornstarch products with</p> <p>23 ovarian cancer?</p> <p>24 MS. CURRY:</p>

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<p style="text-align: right;">Page 198</p> <p>1 Object to the form.</p> <p>2 A Therapeutically or just accidentally?</p> <p>3 MS. THOMPSON:</p> <p>4 Q Um -- as a substitute for talcum</p> <p>5 powder. If a woman is using corn -- a</p> <p>6 cornstarch-based perineal dusting powder, are you</p> <p>7 aware of any studies that have linked that usage</p> <p>8 to ovarian cancer?</p> <p>9 A Not that I -- no.</p> <p>10 Q Do you agree that -- I might go ahead</p> <p>11 and go back to that -- that -- the FDA, mark it</p> <p>12 as --</p> <p>13 A The letter?</p> <p>14 Q The letter.</p> <p>15 I know. But I don't have my stickers.</p> <p>16 MS. THOMPSON:</p> <p>17 My fault; not yours.</p> <p>18 THE COURT REPORTER:</p> <p>19 Okay.</p> <p>20 MS. THOMPSON:</p> <p>21 Shall we do another few just to get us</p> <p>22 to lunch?</p> <p>23 THE COURT REPORTER:</p> <p>24 I forget what number we're on.</p>	<p style="text-align: right;">Page 200</p> <p>1 summary on the following page, one, purpose and</p> <p>2 coverage of the final rule, and the last</p> <p>3 paragraph -- or the last sentence of the first</p> <p>4 paragraph says, "However, the use of powder on</p> <p>5 medical gloves presents numerous risks to</p> <p>6 patients and healthcare workers, including</p> <p>7 inflammation, granulomas and respiratory allergic</p> <p>8 reaction."</p> <p>9 Does that at least state what the FDA</p> <p>10 considers the reasons for the removal of talcum</p> <p>11 powder from surgical gloves?</p> <p>12 A Yes, it does.</p> <p>13 Q Are you aware that Health Canada</p> <p>14 determined that the migration of talc particles</p> <p>15 to the ovaries from perineal use was a plausible</p> <p>16 or is a plausible mechanism for the detection of</p> <p>17 talc in the ovaries?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A I believe they did. You're --</p> <p>21 MS. THOMPSON:</p> <p>22 Q And you -- do you disagree with the</p> <p>23 determination that Health Canada reached</p> <p>24 regarding the -- the migration of talc particles</p>
<p style="text-align: right;">Page 199</p> <p>1 MS. THOMPSON:</p> <p>2 We're on --</p> <p>3 MS. EVERETT:</p> <p>4 14.</p> <p>5 MS. THOMPSON:</p> <p>6 14.</p> <p>7 (DEPOSITION NUMBER 14 WAS</p> <p>8 MARKED FOR IDENTIFICATION.)</p> <p>9 MS. THOMPSON:</p> <p>10 Q I'm going to go ahead and mark the FDA</p> <p>11 announcement on the banning of -- of talcum</p> <p>12 powder just so we can see what they actually did</p> <p>13 say about the reasons.</p> <p>14 And --</p> <p>15 A This is for gloves. For gloves.</p> <p>16 Surgical gloves.</p> <p>17 Q Examination and surgical gloves.</p> <p>18 A Yeah.</p> <p>19 Q And just in the bottom part of the</p> <p>20 right-hand side of the first page, "Banned</p> <p>21 Devices; Powdered Surgeon's Gloves, Powdered</p> <p>22 Patient Examination Gloves, and Absorbable Powder</p> <p>23 For Lubricating on a Surgeon's Glove."</p> <p>24 And if you'll turn to the executive</p>	<p style="text-align: right;">Page 201</p> <p>1 to the ovaries being a plausible mechanism for</p> <p>2 the detection of talc in ovaries?</p> <p>3 A Yes, I do.</p> <p>4 Q In your report, you state that the</p> <p>5 migration is contrary to basic anatomy and common</p> <p>6 sense, I believe.</p> <p>7 Do you still hold that opinion?</p> <p>8 A Where are you reading? Back to my</p> <p>9 report?</p> <p>10 Q I have to get your report out.</p> <p>11 A Yeah. That's get that out there.</p> <p>12 Q His expert report.</p> <p>13 And in the -- under "Migration" on page</p> <p>14 5, "Supposed Presence of Talc in Ovaries."</p> <p>15 A Ah. Okay. Yep.</p> <p>16 Q And Health Canada's conclusion was that</p> <p>17 the migration of talc particles to the ovaries</p> <p>18 from perineal use is a plausible mechanism for</p> <p>19 the detection of talc to the ovaries.</p> <p>20 But at least your opinion is that the</p> <p>21 presence of talc in the ovaries cannot be</p> <p>22 explained by migration. Is that right?</p> <p>23 A Well, the studies that I looked at here</p> <p>24 mostly are the presence of talc in cancer of the</p>

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<p style="text-align: right;">Page 202</p> <p>1 ovary, and there were some control patients, I</p> <p>2 believe, with breast cancer where they looked at</p> <p>3 the ovary.</p> <p>4 And these -- these studies have been</p> <p>5 around for a while. I've reviewed them multiple</p> <p>6 times, and they're just seriously flawed, from my</p> <p>7 perspective. So I don't know that you can</p> <p>8 conclude that. But these are -- these are just</p> <p>9 the studies that show the presence of talc in</p> <p>10 specimens. It's not the next line of evidence,</p> <p>11 which is actual variety of human -- human</p> <p>12 experiments, if you will, which are also</p> <p>13 seriously flawed.</p> <p>14 So, you know, I essentially reviewed</p> <p>15 all of that and came to the conclusion you can't</p> <p>16 conclude anything. There's no convincing data.</p> <p>17 Health Canada came to a different conclusion.</p> <p>18 Q And is that because Health Canada got</p> <p>19 it wrong again, or is that because scientists can</p> <p>20 come to different conclusions when reviewing the</p> <p>21 same data?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A Based on my review on this, they got it</p>	<p style="text-align: right;">Page 204</p> <p>1 A I think they were mystified and they</p> <p>2 tried to argue that the reason why they found</p> <p>3 talc in everybody --</p> <p>4 MS. THOMPSON:</p> <p>5 Q Dr. Birrer, sorry.</p> <p>6 My question was: Do you know what the</p> <p>7 authors concluded?</p> <p>8 A I'm saying it.</p> <p>9 Q That's "yes" or "no."</p> <p>10 A Oh.</p> <p>11 Q Do you know what the authors concluded?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A Yes.</p> <p>15 MS. THOMPSON:</p> <p>16 Q What did the authors conclude?</p> <p>17 A So I think they were mystified. And</p> <p>18 so --</p> <p>19 Q No. Did the authors -- where do you</p> <p>20 see in the paper that the authors were mystified?</p> <p>21 A Because --</p> <p>22 MS. CURRY:</p> <p>23 Let him finish and don't cut him off.</p> <p>24 MS. THOMPSON:</p>
<p style="text-align: right;">Page 203</p> <p>1 wrong.</p> <p>2 MS. THOMPSON:</p> <p>3 Q Regarding the Heller paper --</p> <p>4 A Uh-huh.</p> <p>5 Q -- let's just go back to your report.</p> <p>6 Do you know what the Heller authors</p> <p>7 concluded from their study?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Do you --</p> <p>11 MS. THOMPSON:</p> <p>12 Q This is the paper regarding the talc</p> <p>13 presence in --</p> <p>14 A Right.</p> <p>15 Q -- ovaries from the Heller paper.</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A So just to summarize real quick --</p> <p>19 MS. THOMPSON:</p> <p>20 Q No. Not asking that question.</p> <p>21 Do you know what the Heller authors</p> <p>22 concluded on the basis of their study?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p style="text-align: right;">Page 205</p> <p>1 Not when he's not answering my</p> <p>2 question.</p> <p>3 THE WITNESS:</p> <p>4 Well, I --</p> <p>5 MS. CURRY:</p> <p>6 He's trying to answer it. You keep</p> <p>7 cutting him off at every word.</p> <p>8 MS. THOMPSON:</p> <p>9 I asked where in the paper did the</p> <p>10 authors say they were mystified, and he needs to</p> <p>11 explain that.</p> <p>12 MS. CURRY:</p> <p>13 You haven't even marked the paper. You</p> <p>14 are asking him based on his expert report, and</p> <p>15 he's --</p> <p>16 MS. THOMPSON:</p> <p>17 I didn't ask him on the basis of his</p> <p>18 expert report. I asked him on the basis of his</p> <p>19 knowledge.</p> <p>20 I'll mark the Heller paper 15.</p> <p>21 (DEPOSITION EXHIBIT NUMBER 15 WAS</p> <p>22 MARKED FOR IDENTIFICATION.)</p> <p>23 MS. THOMPSON:</p> <p>24 Q Do you see anywhere in the paper that</p>

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<p style="text-align: right;">Page 206</p> <p>1 the authors were mystified? Yes or no?</p> <p>2 A I think they were confused by the lack</p> <p>3 of association.</p> <p>4 Q Do you see where the authors were</p> <p>5 mystified?</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 MS. THOMPSON:</p> <p>9 Q There's nowhere where the authors say</p> <p>10 they were mystified, is there, Dr. Birrer?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 MS. THOMPSON:</p> <p>14 Q I'll withdraw the question.</p> <p>15 A Okay.</p> <p>16 Q Let's just go to the conclusions.</p> <p>17 "Conclusions: The detection of talc in</p> <p>18 all ovaries demonstrates that it can reach the</p> <p>19 upper genital tract."</p> <p>20 Is that what the authors of the Heller</p> <p>21 paper conclude?</p> <p>22 A Yes.</p> <p>23 Q And yet you're critical of the</p> <p>24 plaintiffs' experts because they conclude the</p>	<p style="text-align: right;">Page 208</p> <p>1 Q Is that your opinion?</p> <p>2 A Say that again.</p> <p>3 Q It's not that scientists can come to</p> <p>4 different conclusions. It's that the 12 experts</p> <p>5 who state the same conclusions as the authors of</p> <p>6 the paper are wrong and you're right?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 MS. THOMPSON:</p> <p>10 Q Is that a correct statement?</p> <p>11 A Correct.</p> <p>12 Q One of your criticisms of the Cramer</p> <p>13 paper from 2007 that detected talc in lymph nodes</p> <p>14 was that it was a case report; correct?</p> <p>15 A Correct.</p> <p>16 Q And you've published with Dr. Cramer;</p> <p>17 correct?</p> <p>18 A I don't think I'm on papers with</p> <p>19 Dr. Cramer.</p> <p>20 Q And have you seen the paper that was</p> <p>21 published recently of a series of cases in which</p> <p>22 talc was detected in the lymph nodes?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>
<p style="text-align: right;">Page 207</p> <p>1 same thing that the authors of the paper</p> <p>2 conclude; right?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 MS. THOMPSON:</p> <p>6 Q In fact, I -- well, go ahead and</p> <p>7 answer.</p> <p>8 A Well, I'm critical of the paper and the</p> <p>9 experts who agreed with it.</p> <p>10 Q And I -- I think there were no fewer</p> <p>11 than 12 experts that you think were wrong on</p> <p>12 this; right?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A If that's the number of experts that</p> <p>16 agreed to it, then, yeah. I agree on that.</p> <p>17 MS. THOMPSON:</p> <p>18 Q And it's not that scientists can come</p> <p>19 to different conclusions. It's that 12 experts</p> <p>20 who state the same conclusions as the authors of</p> <p>21 the paper are wrong and you're right?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 209</p> <p>1 A Do you have an author?</p> <p>2 MS. THOMPSON:</p> <p>3 Q Same authors.</p> <p>4 A So Dr. Cramer --</p> <p>5 Q The lead author is McDonald, but from</p> <p>6 Cramer's lab --</p> <p>7 A I have seen it.</p> <p>8 Q -- and Welch. You've seen it?</p> <p>9 A Uh-huh.</p> <p>10 Q And is it your understanding that the</p> <p>11 authors -- I'll mark the McDonald paper Exhibit</p> <p>12 16.</p> <p>13 (DEPOSITION EXHIBIT NUMBER 16 WAS</p> <p>14 MARKED FOR IDENTIFICATION.)</p> <p>15 MS. THOMPSON:</p> <p>16 Q Is it your understanding that the</p> <p>17 authors specifically controlled for any</p> <p>18 possibility of contamination?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A No. That's not my understanding.</p> <p>22 MS. THOMPSON:</p> <p>23 Q Well, it's in the abstract, if we can</p> <p>24 get -- delve deeper if we need to. The authors</p>

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<p>1 said that since talc can be a surface contaminant</p> <p>2 from tissue collection preparation, digestion</p> <p>3 measurements may be influenced by contamination.</p> <p>4 Instead, because they preserve anatomic landmarks</p> <p>5 and permit identification of particles in cells</p> <p>6 and tissues polarized light microscopy and in</p> <p>7 situ SEM-EDX are recommended to assess talc in</p> <p>8 lymph nodes.</p> <p>9 And that's the methodology that the</p> <p>10 authors, the researchers, performed to assure</p> <p>11 themselves that this finding was not due to</p> <p>12 contamination; right?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A You are reading correctly.</p> <p>16 MS. THOMPSON:</p> <p>17 Q I didn't even read that.</p> <p>18 A Oh.</p> <p>19 Q I came up with that --</p> <p>20 A Oh. I thought you were looking at the</p> <p>21 paper.</p> <p>22 Q Well, I must be right, then.</p> <p>23 A I mean, they -- they observe -- I read</p> <p>24 this -- I'll read it. "In conclusion, talc</p>	<p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A So they -- they observe -- they observe</p> <p>4 large amounts of contamination. They argue that</p> <p>5 with their technology, they can tell whether some</p> <p>6 is surface and some is internal, in lymph nodes.</p> <p>7 MS. THOMPSON:</p> <p>8 Q And they determined that some was</p> <p>9 internal; right?</p> <p>10 A I believe so.</p> <p>11 Q Probably have another, what, five</p> <p>12 minutes and then lunch, or I can do it after we</p> <p>13 come back.</p> <p>14 MS. CURRY:</p> <p>15 Is that okay with you?</p> <p>16 A That's okay.</p> <p>17 MS. CURRY:</p> <p>18 Is that okay with the court reporter?</p> <p>19 THE COURT REPORTER:</p> <p>20 That's fine. Yes.</p> <p>21 THE WITNESS:</p> <p>22 You all right? I'll stop mumbling.</p> <p>23 MS. THOMPSON:</p> <p>24 Q Okay. I want to go over just a few of</p>
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<p>1 contamination in the surface of surgical</p> <p>2 pathology specimens of is common."</p> <p>3 Q Except -- and I didn't have a question</p> <p>4 on the table.</p> <p>5 A Okay.</p> <p>6 Q So I'll object to that as being</p> <p>7 nonresponsive to a question.</p> <p>8 Except the whole purpose of this study</p> <p>9 was to, number one, expand on the case report</p> <p>10 that was published earlier; right?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A I don't see that. It's another study.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Okay.</p> <p>16 A Yeah.</p> <p>17 Q But this had a series of 22 cases;</p> <p>18 right?</p> <p>19 A Twenty-two cases, correct.</p> <p>20 Q And -- and the authors concluded that</p> <p>21 by -- by using the techniques that they used in</p> <p>22 this pap- -- in this paper, they could confirm</p> <p>23 that the -- the talc in the lymph nodes was not</p> <p>24 surface contamination. Right?</p>	<p>1 your criticisms of plaintiffs' experts. And</p> <p>2 let's start with Dr. Clarke-Pearson. I believe</p> <p>3 that you have met Dr. Clarke-Pearson and know him</p> <p>4 by reputation, at least; correct?</p> <p>5 A I have.</p> <p>6 Q He's a past president, I believe, of</p> <p>7 SGO; correct?</p> <p>8 A Correct.</p> <p>9 Q And department chair at University of</p> <p>10 North Carolina, recently retired; correct?</p> <p>11 A Correct.</p> <p>12 Q And -- and you actually wrote the</p> <p>13 criticism here of Dr. Clarke-Pearson?</p> <p>14 A Correct.</p> <p>15 Q And that's your language?</p> <p>16 A Uh-huh.</p> <p>17 Q Okay. Let's just read through that.</p> <p>18 "Dr. Clarke-Pearson analogizes to the migration</p> <p>19 of sperm" -- and this is considering the</p> <p>20 migration of talc particles -- "into tubes after</p> <p>21 coitus. It is rather surprising to hear this</p> <p>22 from a gynecological oncologist."</p> <p>23 Did you look at Dr. Clarke-Pearson's</p> <p>24 references?</p>

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<p style="text-align: right;">Page 214</p> <p>1 A I looked at his expert report.</p> <p>2 Q Including his references?</p> <p>3 A I probably would have paged through it,</p> <p>4 yeah. Yep.</p> <p>5 Q "The obvious difficulty with this line</p> <p>6 of reasoning is the fact that spermatozoa are</p> <p>7 motile and have evolved under millions of years</p> <p>8 to be able to migrate under their own control to</p> <p>9 increase the potential to fertilize the egg.</p> <p>10 This mode of transport is not consistent with a</p> <p>11 talc particle."</p> <p>12 Did you look at Dr. Pearson's citation</p> <p>13 that describes the movement of dead sperm and</p> <p>14 talc particles through that upper genital tract?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A Yeah. I didn't see the -- I didn't see</p> <p>18 the reference on dead sperm. But --</p> <p>19 MS. THOMPSON:</p> <p>20 Q If -- if there was a reference that</p> <p>21 dead sperm moved through and moved through quite</p> <p>22 easily, then your statement that it's not</p> <p>23 analogous because spermatozoa are motile is</p> <p>24 incorrect, isn't it?</p>	<p style="text-align: right;">Page 216</p> <p>1 A Are they dead dead or --</p> <p>2 Q Do you think dead sperm may be motile?</p> <p>3 Do you know any -- too much about reproductive</p> <p>4 physiology?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A A fair amount, yeah.</p> <p>8 MS. THOMPSON:</p> <p>9 Q And you don't know whether dead sperm</p> <p>10 would be motile or not?</p> <p>11 A So how are you defining that?</p> <p>12 They're -- they're -- they've decayed? They're</p> <p>13 broken down --</p> <p>14 Q Yes.</p> <p>15 A -- or the flagella is not moving?</p> <p>16 Q The flagella is not moving in a dead</p> <p>17 sperm.</p> <p>18 A Okay.</p> <p>19 Q Is it?</p> <p>20 A I guess as you are specifically</p> <p>21 defining --</p> <p>22 Q Are you arguing me -- with me?</p> <p>23 A Can I answer?</p> <p>24 MS. CURRY:</p>
<p style="text-align: right;">Page 215</p> <p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A Well, I have to see the paper, and I</p> <p>4 don't know the details.</p> <p>5 MS. THOMPSON:</p> <p>6 Q Assume with me that there is evidence</p> <p>7 published in the peer-reviewed literature that</p> <p>8 dead sperm and sperm particles move through the</p> <p>9 upper genital tract, then your statement that</p> <p>10 it's not analogous because spermatozoa are motile</p> <p>11 would be incorrect; right?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A So these sperm would be put on the</p> <p>15 perineum like a dusting?</p> <p>16 MS. THOMPSON:</p> <p>17 Q No.</p> <p>18 A Okay.</p> <p>19 Q I'm just saying it's -- your statement</p> <p>20 that that is the reason would be incorrect.</p> <p>21 A I -- so --</p> <p>22 Q Are -- are dead sperm motile?</p> <p>23 A I don't actually know. They --</p> <p>24 Q You're --</p>	<p style="text-align: right;">Page 217</p> <p>1 I'm sorry. You can each just take</p> <p>2 turns. Just please let her get her question out.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Do you not know whether dead sperm</p> <p>5 would be motile or not?</p> <p>6 A I would think most of the time they</p> <p>7 would not be motile.</p> <p>8 Q Okay. And would you agree that a sperm</p> <p>9 particle -- for example, if the flagellum is</p> <p>10 broken off, would you agree that would not be</p> <p>11 motile, a sperm particle?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A Motile, moving under its own --</p> <p>15 MS. THOMPSON:</p> <p>16 Q Moving on its own.</p> <p>17 A Yeah. I think it's unlikely.</p> <p>18 Q Do you know the size of the head of a</p> <p>19 sperm?</p> <p>20 A No.</p> <p>21 Q If the reason that Dr. Clarke-Pearson</p> <p>22 was incorrect referencing dead and -- dead sperm</p> <p>23 and sperm particles moving through the upper</p> <p>24 genital tract could be relevant to a talc</p>

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<p style="text-align: right;">Page 218</p> <p>1 particle. If your reason for saying that opinion 2 is incorrect is that sperm are motile, then that 3 reasoning is incorrect, isn't it? 4 MS. CURRY: 5 Object to the form. 6 A Well, I think in the way it's expressed 7 here, that, obviously, it doesn't mean -- I mean, 8 it makes no sense to apply to spermatozoa, which 9 are mobile. But if you're telling me there's a 10 reference for dead sperm, then the question 11 becomes what's in that reference? So these -- 12 MS. THOMPSON: 13 Q Okay. 14 A -- dead sperm were deposited into the 15 uterus after coitus and -- 16 Q We're just talking -- we're not talking 17 about coitus. 18 Is it plausible to you -- 19 A Okay. 20 Q -- that a woman who has talcum on her 21 perineum -- 22 A Uh-huh. 23 Q -- could have coitus and the talcum 24 powder on the perineum could be placed in the</p>	<p style="text-align: right;">Page 220</p> <p>1 Object to the form. 2 A Yeah, I don't know what -- 3 MS. THOMPSON: 4 Q Those are your words. Are 5 Dr. Clarke-Pearson's opinions contrary to 6 knowledge of basic anatomy? 7 MS. CURRY: 8 Object to the form. 9 A Where are you reading? 10 MS. THOMPSON: 11 Q Well, for right now I was just in the 12 first paragraph of "Hypothesized migration of 13 talc to ovaries." 14 A What page? Is it on my report? 15 Q Page 7. 16 A Okay. 17 Oh. So you're relating that statement 18 to Clarke-Pearson? 19 Q Well, I believe you say that all the 20 experts have -- have a theory that's contrary to 21 basic anatomy and common sense. 22 A No. What that refers to, I think, is 23 the fact that you're putting -- you're dusting 24 the perineum many times, most of the times, in a</p>
<p style="text-align: right;">Page 219</p> <p>1 vagina forcefully? Is that plausible? 2 A I don't have any data on that. 3 Q Do you have to have data to say whether 4 or not that's plausible? 5 A I am a scientist. 6 Q Well, maybe take off your scientist 7 hat. Is it plausible that a woman who has talcum 8 powder on her perineum and has sex, that the 9 talcum powder could be forced into the vagina? 10 MS. CURRY: 11 Object to the form. 12 MS. THOMPSON: 13 Q Is it plausible? 14 A Sexual intercourse? 15 Q Sexual intercourse, yes. 16 A Yes. Just getting specifics. 17 Yeah. I mean, I -- I think the way 18 you're hypothesizing it, I suppose there's a 19 possibility. 20 Q So if those things are possible and 21 plausible, then you really don't think 22 Dr. Clarke-Pearson's opinions are unreasonable 23 and -- and are contrary to basic anatomy, do you? 24 MS. CURRY:</p>	<p style="text-align: right;">Page 221</p> <p>1 woman who's vertical, and this concept is that 2 somehow that talc and dust essentially ascends 3 into the ovary. And I think that more often than 4 not lacks common sense and basic anatomy because 5 of what I just said. 6 Now, if you want to go through each 7 individual study, I'm happy to do that because 8 there are methodologic flaws in them. But that 9 statement does not relate directly to 10 Dr. Clarke-Pearson. If it did, it would be under 11 his name. 12 Q But you talk generally about 13 plaintiffs' experts, too. And do you think that 14 you have a better understanding of female anatomy 15 than Dr. Clarke-Pearson? 16 MS. CURRY: 17 Object to the form. 18 A Dr. Clarke-Pearson's pretty good with 19 female anatomy. 20 MS. THOMPSON: 21 Q Do you think you have a better 22 understanding than Dr. Clarke-Pearson of female 23 reproductive physiology? 24 MS. CURRY:</p>

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<p style="text-align: right;">Page 222</p> <p>1 Object to the form.</p> <p>2 A No. I think he would be more versed in</p> <p>3 that.</p> <p>4 MS. THOMPSON:</p> <p>5 Q And -- and you've just testified that</p> <p>6 we're not just talking about a woman standing up</p> <p>7 and putting dusting powder and the ascension. We</p> <p>8 are talking about the possibility, in your words,</p> <p>9 that powder could be on the perineum and</p> <p>10 introduced in the vagina forcefully with sexual</p> <p>11 intercourse; right?</p> <p>12 A Well, yes --</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A We just had that conversation. I mean,</p> <p>16 again, it's hypothetical. Yeah.</p> <p>17 MS. THOMPSON:</p> <p>18 Q Okay. Agreed. I mean, I agree that's</p> <p>19 your opinion.</p> <p>20 And how about a woman who applies</p> <p>21 talcum powder to a sanitary napkin? Is it</p> <p>22 possible that the talcum powder would be</p> <p>23 introduced in the vagina through menstrual flow?</p> <p>24 A Through menstrual --</p>	<p style="text-align: right;">Page 224</p> <p>1 Q Do you think he would know it, what's</p> <p>2 published in literature?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A He might.</p> <p>6 MS. THOMPSON:</p> <p>7 Q So you're certainly not opining today</p> <p>8 that you have a better understanding than</p> <p>9 Dr. Clarke-Pearson of materials that can travel</p> <p>10 retrograde through the upper genital tract, do</p> <p>11 you?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A Oh, I disagree with that.</p> <p>15 MS. THOMPSON:</p> <p>16 Q You think you do have a better</p> <p>17 understanding than Dr. Clarke-Pearson regarding</p> <p>18 whether or not particles can travel through the</p> <p>19 upper genital tract?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A Based upon my analysis of these papers,</p> <p>23 yes.</p> <p>24 MS. THOMPSON:</p>
<p style="text-align: right;">Page 223</p> <p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A Not that I know of. I don't have any</p> <p>4 data for that.</p> <p>5 MS. THOMPSON:</p> <p>6 Q Is that -- you don't think it's</p> <p>7 possible?</p> <p>8 A Again, from -- from -- it's</p> <p>9 interesting. So if menstrual flow coming out of</p> <p>10 the vagina with a sanitary napkin, the talc then</p> <p>11 gets into the vagina up to the ovaries. It</p> <p>12 doesn't make a lot of sense to me.</p> <p>13 Q What percentage of women have</p> <p>14 retrograde menstruation on a -- on a given</p> <p>15 period?</p> <p>16 A I don't understand what you mean by</p> <p>17 that.</p> <p>18 Q Do you think Dr. Clarke-Pearson</p> <p>19 probably knows that percentage?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A I'm sure he'd probably have an opinion</p> <p>23 on it.</p> <p>24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 225</p> <p>1 Q Well, you certainly didn't know about</p> <p>2 dead sperm and sperm particles, did you?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A Well, it's one paper.</p> <p>6 MS. THOMPSON:</p> <p>7 Q And you don't know about -- you don't</p> <p>8 know how many -- what percentage of women have</p> <p>9 retrograde menstruation, which is a classic paper</p> <p>10 in gynecology -- gynecology? You don't know that</p> <p>11 percentage, do you?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A I can't quote you that percentage.</p> <p>15 MS. THOMPSON:</p> <p>16 Q Do you know that women oftentimes use</p> <p>17 baby powder at bedtime?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A I guess that's possible.</p> <p>21 MS. THOMPSON:</p> <p>22 Q And that would not be in an upright</p> <p>23 position, would it?</p> <p>24 MS. CURRY:</p>

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<p style="text-align: right;">Page 226</p> <p>1 Object to the form.</p> <p>2 A They may have put it on in an upright</p> <p>3 position.</p> <p>4 MS. THOMPSON:</p> <p>5 Q And do you agree that women could have</p> <p>6 powder on the perineum and use a tampon?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A I assume that's possible, yes.</p> <p>10 MS. THOMPSON:</p> <p>11 Q And wouldn't it be possible that powder</p> <p>12 on a tampon could be introduced into the vagina?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A It's possible.</p> <p>16 MS. THOMPSON:</p> <p>17 Q And what -- what did Dr. Kunz, K-U-N-Z,</p> <p>18 describe in an article regarding how particles</p> <p>19 and substances are transported to the upper</p> <p>20 genital tract?</p> <p>21 A So that's the peristaltic pump.</p> <p>22 Q And describe that for me.</p> <p>23 A Yeah. So they went and looked at the</p> <p>24 contractions -- they, first of all, tried to</p>	<p style="text-align: right;">Page 228</p> <p>1 Object to the form.</p> <p>2 A Yeah.</p> <p>3 The problem I have with that is I'm not</p> <p>4 sure what direction the pressure is in, because</p> <p>5 obviously if you give oxytocin at the time of</p> <p>6 pregnancy after the delivery, expels the</p> <p>7 placenta, so some of that pressure's going to</p> <p>8 come down.</p> <p>9 And, then, too, the radioactive studies</p> <p>10 are really problematic because a lot of times the</p> <p>11 label will come off of the microsphere. So you</p> <p>12 don't quite know where it's going.</p> <p>13 MS. THOMPSON:</p> <p>14 Q At what points in a female's -- in a</p> <p>15 woman's cycle are oxytocin levels the highest?</p> <p>16 A I can't quote you that.</p> <p>17 Q Would that be a question for</p> <p>18 Dr. Clarke-Pearson?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A He probably would know.</p> <p>22 MS. THOMPSON:</p> <p>23 Q And are you aware of the studies</p> <p>24 showing that not only sperm particles and dead</p>
<p style="text-align: right;">Page 227</p> <p>1 measure the pressure in the uterus based on this</p> <p>2 contraction, and they used actually ultrasound to</p> <p>3 do it, which is an indirect measure, of course.</p> <p>4 Don't know really what the pressure is.</p> <p>5 Based upon finding that, then they went</p> <p>6 on to, if I recall correctly, use micro- --</p> <p>7 radiolabeled microspheres to do -- a word I can't</p> <p>8 pronounce -- hysterosalpingoscintigraphy,</p> <p>9 whatever.</p> <p>10 Q I can't either.</p> <p>11 A Yeah. And the idea was -- if I recall</p> <p>12 correctly, the idea of that whole study was</p> <p>13 actually for -- I think fertility and pregnancy.</p> <p>14 And the idea was that they then saw this</p> <p>15 radioactivity up in the areas and drew the</p> <p>16 conclusion that there is contraction to the</p> <p>17 uterus and that they were hypothesizing that the</p> <p>18 particles then were going up the tubes of the</p> <p>19 ovaries.</p> <p>20 Q So it facilitates movement through</p> <p>21 the --</p> <p>22 A Yeah.</p> <p>23 Q -- genital tract?</p> <p>24 MS. CURRY:</p>	<p style="text-align: right;">Page 229</p> <p>1 sperm move through the upper genital tract but</p> <p>2 even motile sperm move at a much faster rate than</p> <p>3 would be predicted strictly based on their</p> <p>4 self-generated motility?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A Yeah. I actually recall seeing that in</p> <p>8 a study.</p> <p>9 MS. THOMPSON:</p> <p>10 Q Are you aware that motile sperm</p> <p>11 preferentially go to the side where ovulation has</p> <p>12 occurred?</p> <p>13 A That, I'm not -- I can't quote you</p> <p>14 that. I don't know.</p> <p>15 Q So that would probably be another</p> <p>16 question for one of the gynecologists or --</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 MS. THOMPSON:</p> <p>20 Q -- gynecologic oncologists? Would you</p> <p>21 agree?</p> <p>22 A They -- they would have that, and their</p> <p>23 OB training would provide them with that</p> <p>24 information. Yeah.</p>

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<p style="text-align: right;">Page 230</p> <p>1 Q Let's break for lunch. 2 VIDEOGRAPHER: 3 Off the record at 12:55 p.m. 4 (Lunch recess.) 5 VIDEOGRAPHER: 6 We're back on the record at 2:02 p.m. 7 MS. THOMPSON: 8 Q Dr. Birrer, I think we established this 9 morning that it is your opinion that the genital 10 use of talcum powder is not a risk factor for 11 ovarian cancer; right? 12 A I'm sorry. Say that -- say that again. 13 Q It's your opinion that talcum powder is 14 not a risk factor for ovarian cancer; right? 15 A The use of talcum powder? 16 Q Yes. 17 A Correct. 18 Q Can you point me to any article -- can 19 you point me to an article that specifically 20 states genital talcum powder use is not a risk 21 factor for -- for ovarian cancer? 22 MS. CURRY: 23 Object to the form. 24 A That genital talcum powder use is not a</p>	<p style="text-align: right;">Page 232</p> <p>1 study? 2 MS. CURRY: 3 Object to the form. 4 A No. I'd have to go through them. Do 5 you have them? 6 MS. THOMPSON: 7 Q We're not gonna go through the 40 8 studies, but -- 9 At least sitting here today, you can't 10 think of one right offhand, can you? 11 A I'm happy to go through the studies. 12 Q Okay. Is it your opinion that genital 13 talcum powder use has been proven to be a safe 14 practice? 15 MS. CURRY: 16 Object to the form. 17 A We discussed that this morning. There 18 is no data I know that it's an unsafe practice. 19 That's a review of the literature. And, so, 20 it's -- I think in that context it's safe. 21 MS. THOMPSON: 22 Q In your previous -- or did you look at 23 websites when you prepared your report this time 24 regarding talcum powder exposure and the risk for</p>
<p style="text-align: right;">Page 231</p> <p>1 risk factor? I mean, if you look at the -- a lot 2 of the case-control studies, about 40 percent of 3 them are negative and -- 4 MS. THOMPSON: 5 Q Well -- and by negative, you mean not 6 statistically significant; right? 7 A (Nods affirmatively.) Negative. And 8 cohort studies aren't either. And -- and, 9 actually, that -- and the cohort studies have 10 been sort of analyzed, reanalyzed in multiple 11 meta-analysis, and so they're all negative. 12 Q But my question was: Did any of those 13 studies conclude talcum powder is not a risk 14 factor for ovarian cancer? 15 MS. CURRY: 16 Object to the form. 17 A So there are studies that don't show a 18 significant association between talcum use and -- 19 MS. THOMPSON: 20 Q But I'm looking for -- 21 A -- and ovarian cancer. 22 Q -- the statement that genital use of 23 talcum is not a risk factor for ovarian cancer. 24 Do you remember seeing that in any</p>	<p style="text-align: right;">Page 233</p> <p>1 ovarian cancer? 2 MS. CURRY: 3 Object to the form. 4 A Other than PubMed? 5 MS. THOMPSON: 6 Q Right. 7 Like the American Cancer Society or NCI 8 or any websites. 9 A Not for this one. 10 Q Had you looked at them before? 11 MS. CURRY: 12 Object to the form. 13 A I think in the previous depositions, I 14 reported looking at one or two of them. I'd have 15 to go back and look at that. 16 MS. THOMPSON: 17 Q Okay. 18 A Yeah. 19 Q And I think the American Cancer Society 20 website was one of those that you looked at. 21 Correct? 22 A Could be. 23 Q I'll mark 17, American Cancer Society, 24 Talcum Powder and Cancer.</p>

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<p style="text-align: right;">Page 234</p> <p>1 (DEPOSITION EXHIBIT NUMBER 17 2 WAS MARKED FOR IDENTIFICATION.) 3 MS. THOMPSON: 4 Q Does that look familiar? 5 A That looks like American Cancer 6 Society's website. Because I see the logo. 7 Q And -- and would you use this statement 8 on the American Cancer Society website to be 9 support for your opinion that talcum powder use 10 is not a risk factor for ovarian cancer? 11 A Is not a risk factor? Is not? 12 Q Is not. 13 A I wouldn't refer to this, no. 14 Q Do you think that's what this document 15 states? 16 A I don't think this -- it doesn't seem 17 to me, based on what the ACS is saying -- they 18 report that their findings are mixed, with some 19 studies reporting a slightly increased risk and 20 some reporting no increase. 21 Q So the American Cancer Society, on 22 their website, states that IARC has classified 23 talc that contains asbestos as carcinogenic to 24 humans; right?</p>	<p style="text-align: right;">Page 236</p> <p>1 talcum powder does not increase risk, are they? 2 MS. CURRY: 3 Object to the form. 4 A Say again. 5 MS. THOMPSON: 6 Q They're not saying that talcum powder 7 use does not increase cancer risk, do they? 8 A I don't see that stated. 9 Q And -- and they say there is some 10 suggestion of a possible increase in ovarian 11 cancer risk; right? 12 A Well, the statement I see is "It's not 13 clear if consumer products containing talcum 14 increase cancer risks." That's pretty specific. 15 Q They're saying it's not clear. It's 16 not saying it's not a risk, is it? 17 MS. CURRY: 18 Object to the form. 19 A They're saying they don't know. 20 MS. THOMPSON: 21 Q Right. And then the recommendation, by 22 the American Cancer Society, would be "Until more 23 information is available, people concerned about 24 using talcum powder may want to avoid or limit</p>
<p style="text-align: right;">Page 235</p> <p>1 A You're on page 3? 2 Q Yeah. 30 -- yeah, 3 of 6. 3 A Yeah. 4 Q And then based on the lack of data from 5 human studies and unlimited data in lab animal 6 studies, IARC classified inhaled talc not 7 containing asbestos as not classifiable; right? 8 A The second bullet? 9 Q The second bullet. 10 And then the third bullet is the IARC 11 that states that the perineal genital use of talc 12 powder -- talc-based body powder is possibly 13 carcinic- -- carcinogenic to humans. That's the 14 2B classification; right? 15 A 2B. 16 Q And then it states that the US National 17 Toxicology Program, NTB, has not fully reviewed 18 talc with or without asbestos as a possible 19 carcinogen; right? That's what it says. 20 A Correct. 21 Q And, then, as -- as you said, the ACS 22 states it's not clear if consumer products 23 containing talcum powder increase cancer risk. 24 They're certainly not saying that</p>	<p style="text-align: right;">Page 237</p> <p>1 their use of consumer products that contain it." 2 But you think any recommendation of 3 that kind is not indicated; correct? 4 MS. CURRY: 5 Object to the form. 6 A Well, it depends on how you read that. 7 I mean, I think what they're suggesting is that 8 people concerned about using talcum powder, for 9 whatever reason, may want to avoid or limit their 10 use of consumer products that contain it and 11 implies that it's the stress of knowing they're 12 using it because of what they've interpreted. It 13 doesn't really make any conclusions about talcum 14 powder. 15 MS. THOMPSON: 16 Q Are there any medical benefits that 17 you're aware of from the genital use of talcum 18 powder? 19 A Well, I think it's generally used to 20 absorb -- absorb fluid. It's -- a lot of women 21 like it. It's a body image issue. You know, so 22 I think those issues -- and again, I treat a lot 23 of women with ovarian cancer -- are important. 24 Q That wasn't my question.</p>

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<p style="text-align: right;">Page 238</p> <p>1 Are there any medical benefits to the</p> <p>2 genital use of talcum powder?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A That is a medical use?</p> <p>6 MS. THOMPSON:</p> <p>7 Q Are there any benefits, is the</p> <p>8 question.</p> <p>9 A Yeah.</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 MS. THOMPSON:</p> <p>13 Q Where are -- where are those benefits</p> <p>14 reported?</p> <p>15 A That's quality of life.</p> <p>16 Q Where in the medical literature can you</p> <p>17 show a report that describes medical benefits</p> <p>18 from the genital use of talcum powder?</p> <p>19 A Well, it's not in -- and again, I</p> <p>20 didn't review that for this expert report, so --</p> <p>21 but you're asking me.</p> <p>22 Q When you -- if you're trying to make a</p> <p>23 risk assessment, wouldn't you know if you're</p> <p>24 weighing the benefits versus the potential risks?</p>	<p style="text-align: right;">Page 240</p> <p>1 A Again, you asked me the question about</p> <p>2 do I think there's some medical benefit. I --</p> <p>3 the answer is yes. I mean --</p> <p>4 MS. THOMPSON:</p> <p>5 Q But that's never been published</p> <p>6 anywhere that you're aware of, has it?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A As I said before, I -- I can't quote</p> <p>10 you that.</p> <p>11 MS. THOMPSON:</p> <p>12 Q Is it -- have you seen in the medical</p> <p>13 literature that there are no benefits, medical</p> <p>14 benefits from the use of talcum powder in the</p> <p>15 genital area?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A I don't think I've actually seen that.</p> <p>19 MS. THOMPSON:</p> <p>20 Q Would you be surprised if there are</p> <p>21 references in numerous articles that say because</p> <p>22 there are no medical benefits of talcum powder</p> <p>23 use, it's not recommended?</p> <p>24 MS. CURRY:</p>
<p style="text-align: right;">Page 239</p> <p>1 A Well, I evaluated the risks, and there</p> <p>2 are none.</p> <p>3 Q So you just evaluated the risk and</p> <p>4 it -- it wouldn't matter to you whether there</p> <p>5 were benefits or not.</p> <p>6 A Well, my benefit --</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A I'm sorry. Go ahead. I'm sorry.</p> <p>10 Yeah. My benefit would be based upon</p> <p>11 my own experience. It's not necessarily</p> <p>12 published in medical literature.</p> <p>13 MS. THOMPSON:</p> <p>14 Q Okay. Well, that would certainly be</p> <p>15 anecdotal, wouldn't it?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A Well, you know, I've got a lot of</p> <p>19 experience.</p> <p>20 MS. THOMPSON:</p> <p>21 Q It's still anecdotal, isn't it,</p> <p>22 Dr. Birrer?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p style="text-align: right;">Page 241</p> <p>1 Object to the form.</p> <p>2 A I'd be happy to -- I'd be happy to</p> <p>3 review them.</p> <p>4 MS. THOMPSON:</p> <p>5 Q Have you seen in the medical literature</p> <p>6 that cornstarch products are recommended if women</p> <p>7 choose to use a dusting powder over talcum</p> <p>8 powder?</p> <p>9 A Can you repeat that? I -- the cough.</p> <p>10 Q Have you seen in the medical literature</p> <p>11 that -- where cornstarch products are recommended</p> <p>12 if women choose to use a dusting powder over</p> <p>13 talcum powder?</p> <p>14 A You know, I haven't seen the -- I</p> <p>15 haven't seen the medical literature recommending</p> <p>16 cornstarch over talcum. But I have seen -- I've</p> <p>17 seen discussions about women who use cornstarch.</p> <p>18 Q And again, there have never been any</p> <p>19 risks that you're aware of into -- related to the</p> <p>20 genital use of cornstarch products and the link</p> <p>21 with ovarian cancer; right?</p> <p>22 A I don't know of any.</p> <p>23 Q You mentioned earlier this morning the</p> <p>24 National Academy of Science, Engineering and</p>

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<p style="text-align: right;">Page 242</p> <p>1 Medicine as a -- as a -- possibly the most 2 reputable source of credible information. 3 Would -- did I describe that sort of 4 correctly? 5 MS. CURRY: 6 Object to the form. 7 A I don't recall saying it's the most, 8 but I used it in context of comparing IARC, if I 9 recall correctly, versus some other sort of pure 10 scientific professional organization, which I 11 would include the National Academy to be that. 12 MS. THOMPSON: 13 Q Okay. Fair enough. 14 And I'm sure you're familiar with the 15 treatise -- it's actually -- came out in book 16 form -- of the study by the Institute of 17 Medicine, I believe, at that time, on ovarian 18 cancer? 19 A Yes. 20 Q Did you participate at all in that 21 study? 22 A They asked me to review it. 23 Q You were one of the reviewers? 24 A They asked me to review it.</p>	<p style="text-align: right;">Page 244</p> <p>1 Q I'll give it to you in a minute. 2 A Okay. 3 Q I just want to ask you a few questions 4 first. 5 Why did you decline to review? 6 A I was too busy. 7 Q Okay. Because it was a big book? 8 A It's monstrous. 9 Q However, several of the authors have 10 been coauthors with you on -- on papers. Is one 11 of them Dr. Karlan? 12 A I believe I've been on papers with 13 Beth. And I think Anil Sood was on there, too. 14 THE COURT REPORTER: 15 Excuse me? 16 THE WITNESS: 17 Anil Sood, S-O-O-D. 18 MS. THOMPSON: 19 Q And Ronald Alvarez -- Alvarez published 20 with you, I think? 21 A I believe so. 22 Q Dr. Karlan's published with you. 23 A (Nods affirmatively.) 24 Q Dr. Levine has published with you?</p>
<p style="text-align: right;">Page 243</p> <p>1 Q Oh. 2 A I declined. 3 Q They asked you to review it and you did 4 not review it. That explains it, because I 5 didn't see your name on the list. 6 And that was published in 2016? 7 A Uh-huh. 8 Q And what was your understanding of the 9 purpose of that study? 10 MS. CURRY: 11 Object to the form. 12 A It -- I -- you know, I think it was -- 13 this is -- it's just medicine undertakes this 14 periodically for large topics, and that was one 15 of them, to sort of summarize the state of the 16 science. 17 MS. THOMPSON: 18 Q And the -- in fact, the committee that 19 did the study was a committee on the state of the 20 science in ovarian cancer research; is that 21 correct? So you called -- 22 A This is the one by Beth Karlan? 23 Q Yeah. 24 A Yeah.</p>	<p style="text-align: right;">Page 245</p> <p>1 A Doug and I are on a couple of papers, 2 yeah. 3 Q Doug Levine? 4 A Yeah. 5 Q Dr. Odunsi, Kunle Odunsi -- 6 A Kunle. Kunle. 7 Q -- has published with you. And 8 Dr. Sood you mentioned; right? 9 And Dr. -- is it Tworoger or -- 10 A Two- -- Twergger? 11 Q -- Two- -- Twoauger? 12 A T-W-O-G-G-E-R [sic]. 13 Q Has published with you? 14 A I think so, yes. I'd have to check 15 that. 16 Q So you were, I would say, well 17 represented on the -- 18 MS. CURRY: 19 Object to the form. 20 A Well, I know them. 21 MS. THOMPSON: 22 Q -- on the author list? 23 MS. CURRY: 24 Object to the form.</p>

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<p style="text-align: right;">Page 246</p> <p>1 MS. THOMPSON: 2 Q And -- and I assume you would agree 3 with me that the committee to report on the state 4 of the science of ovarian cancer research was 5 selected because of their expertise in the area; 6 correct? 7 A Yes. 8 MS. CURRY: 9 Object to the form. 10 MS. THOMPSON: 11 Q And, as we mentioned, this study was 12 under the auspices of the National Academy of 13 Science, Medicine and Engineering, Institute of 14 Medicine, I believe, originally; correct? 15 A Correct. 16 Q And is it your understanding that this 17 study was also supported by the CDC? 18 A That, I don't know. 19 Q All right. Let me just go ahead and 20 give it to you. 21 A Yeah. 22 (DEPOSITION EXHIBIT NUMBER 18 WAS 23 MARKED FOR IDENTIFICATION.) 24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 248</p> <p>1 A Correct. 2 Q The State of the Science authors state, 3 under "Inflammation," "Studies of the 4 inflammatory marker C-reactive protein suggest a 5 possible association between inflammation and 6 increased risk of ovarian cancer," citing OC and 7 Poole. 8 "Other specific inflammatory factors 9 have also been associated with ovarian cancer." 10 Do you agree that the authors of this 11 treatise reported that there's a possible 12 association between inflammation and increased 13 risk for ovarian cancer? 14 A Well, on these -- on these two 15 sentences, I think they accurately stated, 16 "suggests association." And then they refer -- I 17 don't -- these two papers, I can't directly quote 18 you. I mean -- 19 Q And I -- and I'm not -- 20 A Yeah. 21 Q -- suggesting that they do anything 22 other than suggest the possible association. 23 A Right. 24 Q I'm not trying to read more into it.</p>
<p style="text-align: right;">Page 247</p> <p>1 Q Exhibit 18 I'm marking as Ovarian 2 Cancers, Evolving Paradigms in Research and Care. 3 And this is not the entire book, but it is the 4 entire chapter that we're going to look at, which 5 is "Prevention and Early Detection," Chapter 3. 6 And if you look on page little ix, page 7 9, preface -- 8 A 9? 9? 9 Q Little nine. 10 A Yeah. 11 Q Yeah. The -- the first sentence, "This 12 congressionally mandated report sponsored by the 13 Centers For Disease Control and Prevention 14 assesses the state of research on ovarian cancers 15 from multiple perspectives and by multiple 16 disciplines." 17 So do you agree that the Center For 18 Disease Control sponsored the study? 19 A Correct. 20 Q If you'll turn to page -- I don't have 21 pages on my copy. Page 110. Under the section 22 heading "Inflammation." And this is in a larger 23 section titled "Behavioral and Inflammatory Risk 24 Factors"; correct?</p>	<p style="text-align: right;">Page 249</p> <p>1 A Okay. 2 Q And then they describe "A meta-analysis 3 reported that exposure to asbestos was associated 4 with a 77 percent increased risk of ovarian 5 cancer mortality," citing Carmargo. 6 Are you familiar with that paper? 7 A I am familiar with that. That's the 8 occasional exposure, if I recall correctly. 9 Q And "The International Agency For 10 Research on Cancer determined that there was 11 sufficient evidence to support a causal 12 relationship between asbestos exposure and 13 ovarian cancer." 14 So the authors of this treatise include 15 exposure to asbestos and its association with 16 ovarian cancer in the Inflammation section of -- 17 of risk factors; right? 18 A Say that again? Sorry. For asbestos? 19 Q The authors of this treatise include 20 exposure to asbestos and its association with 21 ovarian cancer in the Inflammation section of 22 risk factors; right? 23 A Correct. 24 Q They go on to say, "This has led to</p>

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<p>1 studies of talc use which is chemically similar</p> <p>2 to asbestos and can cause an inflammatory</p> <p>3 response."</p> <p>4 Do you agree with that statement?</p> <p>5 A I -- I actually hesitate a little on</p> <p>6 that because I'm not so sure that that's a</p> <p>7 temporal relationship, that it was the asbestos</p> <p>8 association that then led to the investigation of</p> <p>9 talc. I don't know, when Dan Cramer published</p> <p>10 his first paper, that's what was driving him.</p> <p>11 Q Do you have any other disagreement with</p> <p>12 the -- the statement other than whether it led to</p> <p>13 the studies of talc use?</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 A I don't know. Again, we've covered</p> <p>17 this. I'm not a mineralogist, so I don't know</p> <p>18 the similarity issues. And inflammatory response</p> <p>19 is not defined. So other than that, it's fine.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Well, the authors -- let's take out the</p> <p>22 asbestos and say "Talc can cause inflammatory</p> <p>23 response." Do you agree or disagree with that?</p> <p>24 A Well, inflammation is a broad issue and</p>	<p>1 one else anywhere in the literature to question</p> <p>2 even this, I don't agree with.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Okay. So you -- so you disagree with</p> <p>5 the authors including that statement in -- in</p> <p>6 this treatise?</p> <p>7 A I just think it's not defined. They</p> <p>8 defined it, then I would have felt a lot better.</p> <p>9 Can cause granulomas inflammatory response. That</p> <p>10 would have been more accurate.</p> <p>11 Q I can understand that you think it</p> <p>12 should have been defined better.</p> <p>13 A Yeah.</p> <p>14 Q But do you agree with the statement</p> <p>15 that's in this treatise, or disagree?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A No opinion.</p> <p>19 MS. THOMPSON:</p> <p>20 Q But you'll agree that at least these</p> <p>21 experts thought it was worthwhile putting the</p> <p>22 statement in this State of the Science treatise</p> <p>23 on ovarian cancer published in 2016; right?</p> <p>24 MS. CURRY:</p>
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<p>1 it's very relevant to this debate, which is are</p> <p>2 we talking granulomas, acute, chronic but</p> <p>3 nongranuloma? I think that's a big issue.</p> <p>4 Q Well, these were the authors that were</p> <p>5 selected because of their expertise to do a State</p> <p>6 of the Science treatise at the behest of the</p> <p>7 National Academy of Science and CDC.</p> <p>8 I'm just asking you do you agree with</p> <p>9 the statement "Talc can cause an inflammatory</p> <p>10 response"?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A And -- and I'm -- I'm answering it.</p> <p>14 MS. THOMPSON:</p> <p>15 Q And you say you don't know? You can't</p> <p>16 agree or disagree? Is that what you're saying?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A The inflammation is not defined. I</p> <p>20 don't know if the similarity between asbestos and</p> <p>21 talc. So other than that, I think it's fine.</p> <p>22 But the -- the -- the implication that all of the</p> <p>23 ovarian cancer experts are on this -- on this --</p> <p>24 on this report and there are no one -- there's no</p>	<p>1 Object to the form.</p> <p>2 A Yeah. Apparently.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Do you know Jason Wright?</p> <p>5 A Division head at Columbia?</p> <p>6 Q Yes.</p> <p>7 A I do know Jason. Not -- I know him by</p> <p>8 reputation. I don't think I've ever actually met</p> <p>9 him.</p> <p>10 Q And what is his reputation?</p> <p>11 A I think he's got a good reputation</p> <p>12 running his division, and he's a good surgeon.</p> <p>13 Q Have you ever published with Jason</p> <p>14 Wright?</p> <p>15 A I don't believe so.</p> <p>16 Q You're right. That was a trick</p> <p>17 question.</p> <p>18 I'm gonna mark --</p> <p>19 MS. CURRY:</p> <p>20 I should have objected.</p> <p>21 (DEPOSITION EXHIBIT NUMBER 19</p> <p>22 WAS MARKED FOR IDENTIFICATION.)</p> <p>23 MS. THOMPSON:</p> <p>24 I'm gonna mark just a short article of</p>

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<p>1 Jason Wright's as Exhibit Number 19.</p> <p>2 Sorry. I thought I gave you mine.</p> <p>3 THE WITNESS:</p> <p>4 We're done with IM?</p> <p>5 MS. THOMPSON:</p> <p>6 Q Yeah, I think so. And this was an</p> <p>7 article published in -- not an article. It's</p> <p>8 a -- under a practice issue, which I think is an</p> <p>9 ongoing column, basically, in The Green Journal.</p> <p>10 What's The Green Journal?</p> <p>11 A OB-GYN, I think?</p> <p>12 Q And is that the journal that -- the</p> <p>13 journal that's published under the ACOG auspices?</p> <p>14 A I believe so.</p> <p>15 Q Are you a member of ACOG?</p> <p>16 A No.</p> <p>17 Q And this was published in December of</p> <p>18 2018, about six months ago. And was titled "Best</p> <p>19 Articles From the Past Year." And the second</p> <p>20 article listed out of four -- and these were</p> <p>21 what's new in ovarian cancer -- is the</p> <p>22 Penninkilampi article published in Epidemiology.</p> <p>23 A Uh-huh.</p> <p>24 Q And Dr. Wright concludes that, bottom</p>	<p>1 THE WITNESS:</p> <p>2 Oh, leaving you in the dust? Sorry.</p> <p>3 And then the use -- UKC talc studies,</p> <p>4 it really pales in comparison because -- and I</p> <p>5 looked at Penninkilampi pretty carefully. It</p> <p>6 kind of revisited all of the previous data. I</p> <p>7 think -- I -- I would assume that Jason doesn't</p> <p>8 necessarily keep up with this literature, so when</p> <p>9 it came out, he looked at it and said, ah, it's a</p> <p>10 meta-analysis. But it doesn't bring much to the</p> <p>11 table, I think.</p> <p>12 MS. THOMPSON:</p> <p>13 Q Well, you're obviously speculating as</p> <p>14 to Dr. Wright's reasoning, because neither --</p> <p>15 neither one of us knows. But at least Dr. Wright</p> <p>16 chose to include this as one of the four best</p> <p>17 articles regarding ovarian cancer in the past</p> <p>18 year published in 2018; right?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A Well, I think he -- I think he -- I</p> <p>22 think he exposed his reasoning a little bit by</p> <p>23 the last sentence in the first paragraph. "The</p> <p>24 possible association with talcum and brain cancer</p>
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<p>1 line, "Perineal application of talc is associated</p> <p>2 with a small increased risk of ovarian cancer."</p> <p>3 Do you disagree with that conclusion by</p> <p>4 Dr. Wright?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A That's his -- I'm trying to figure out</p> <p>8 where you're reading. It's the bottom-line</p> <p>9 statement?</p> <p>10 MS. THOMPSON:</p> <p>11 Q Bottom line, yes.</p> <p>12 A Yeah, I would disagree with that.</p> <p>13 Q Do you disagree with it -- the</p> <p>14 inclusion of the Penninkilampi meta-analysis as</p> <p>15 one of the best articles from the past year?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A You know, it's interesting. I would,</p> <p>19 actually. I -- when -- when you compare it to</p> <p>20 Aerial Three and the Carbon Inhibitors and the</p> <p>21 hypothermic intraperineal chemotherapy, which was</p> <p>22 a New England Journal paper --</p> <p>23 MS. THOMPSON:</p> <p>24 Can you slow down?</p>	<p>1 has attracted media attention, resulting in a</p> <p>2 number of lawsuits."</p> <p>3 So I think that's part of the reason he</p> <p>4 feels this is relevant. Doesn't bring a lot of</p> <p>5 science.</p> <p>6 MS. THOMPSON:</p> <p>7 Q Well, I don't think it was meant to</p> <p>8 bring science. He was choosing this article for</p> <p>9 its -- its relevance for the readership of the</p> <p>10 American College of OB-GYN journal; correct?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A I would agree with that.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Do you have an opinion as to whether</p> <p>16 talc, the mineral talc, is inert?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A You have to define "inert."</p> <p>20 MS. THOMPSON:</p> <p>21 Q Do you have an opinion as to whether</p> <p>22 the mineral talc, if it occurs in pure form --</p> <p>23 I'll add that as well -- is chemically inert?</p> <p>24 MS. CURRY:</p>

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<p style="text-align: right;">Page 258</p> <p>1 Object to the form.</p> <p>2 A Chemically inert, meaning -- again, I'm</p> <p>3 struggling with this, that it -- it -- it can</p> <p>4 enter into chemical reaction with other</p> <p>5 substances.</p> <p>6 MS. THOMPSON:</p> <p>7 Q I'd just seen that phrase used, so I</p> <p>8 wanted to see if you had an understanding of what</p> <p>9 it meant and -- and whether it's -- that</p> <p>10 statement would be true.</p> <p>11 A I really would need -- if -- if you've</p> <p>12 seen it said, do you have it so I can look at it?</p> <p>13 Q I've seen it by your -- your fellow</p> <p>14 experts.</p> <p>15 A And -- and what was the context? There</p> <p>16 must have been a context.</p> <p>17 Q And the context was talc is chemically</p> <p>18 inert. Would you have an opinion on that?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A I think I would say no opinion right</p> <p>22 now.</p> <p>23 MS. THOMPSON:</p> <p>24 Q Okay. Is it biologically inert?</p>	<p style="text-align: right;">Page 260</p> <p>1 MS. CURRY:</p> <p>2 Sorry.</p> <p>3 A That, I don't think I could say with</p> <p>4 confidence.</p> <p>5 MS. THOMPSON:</p> <p>6 Q So even though talc used for</p> <p>7 pleurodesis is biologically -- is not</p> <p>8 biologically inert, you wouldn't be able to say</p> <p>9 whether baby powder was or not?</p> <p>10 A Well, we --</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A Well, we didn't put baby powder into</p> <p>14 the pleural cavities of patients, so we really</p> <p>15 haven't done that.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Would you have any reason to suspect</p> <p>18 that baby powder would behave in a less</p> <p>19 biologically active manner than the talc used in</p> <p>20 pleurodesis?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A Well, the talc -- you know, the talc</p> <p>24 used in pleurodesis is -- and I'm putting</p>
<p style="text-align: right;">Page 259</p> <p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Pure mineral talc. If pure talc</p> <p>5 existed.</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A Huh?</p> <p>9 Okay.</p> <p>10 That's another difficult one. I mean,</p> <p>11 I think that we know talc is used for</p> <p>12 pleurodesis. So that's -- is that a biologic</p> <p>13 process? I think it probably would qualify. So</p> <p>14 I wouldn't call it inert from that standpoint.</p> <p>15 MS. THOMPSON:</p> <p>16 Q And you're not gonna get me to argue</p> <p>17 with that.</p> <p>18 A I don't think so.</p> <p>19 Q Would that opinion apply to Johnson's</p> <p>20 baby powder?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 MS. THOMPSON:</p> <p>24 Q Or do you know?</p>	<p style="text-align: right;">Page 261</p> <p>1 quotations around this -- relatively pure, and</p> <p>2 it's gonna be different than the baby powder.</p> <p>3 But if you're asking me is talc in baby powder, I</p> <p>4 think we can agree on that. And, so, by analogy,</p> <p>5 I would expect some biologic activity.</p> <p>6 MS. THOMPSON:</p> <p>7 Q Okay.</p> <p>8 A Okay.</p> <p>9 Q And same for Shower to Shower?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A Actually don't even know -- I've never</p> <p>13 seen a Shower to Shower container, but it's the</p> <p>14 product; right?</p> <p>15 MS. THOMPSON:</p> <p>16 Q Do you know what's in Shower to Shower?</p> <p>17 A I'm assuming it's analogous to baby</p> <p>18 powder.</p> <p>19 Q If -- well, would -- would that opinion</p> <p>20 apply to fibrous talc?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A You know, again, I'm not a mineralogy</p> <p>24 expert, so I'm not going to make a comment on</p>

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<p style="text-align: right;">Page 262</p> <p>1 that.</p> <p>2 MS. THOMPSON:</p> <p>3 Q Do you know what fibrous talc is?</p> <p>4 A I'm not sure I can really define it.</p> <p>5 Q And it's your understanding that</p> <p>6 fibrous talc or talc with asbestiform fibers is</p> <p>7 specifically excluded from the IARC 2010</p> <p>8 monograph? Correct?</p> <p>9 A Say that again, please.</p> <p>10 Q Is it your -- let me rephrase it just a</p> <p>11 little bit. Is it your understanding that</p> <p>12 fibrous talc or talc with asbestiform fibers is</p> <p>13 specifically excluded from the IARC 2010</p> <p>14 monograph?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A So that's -- asbestiform fibers or</p> <p>18 asbestos?</p> <p>19 MS. THOMPSON:</p> <p>20 Q Asbestiform fibers. Is there a</p> <p>21 difference between fibrous talc and talc with</p> <p>22 asbestiform fibers?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p style="text-align: right;">Page 264</p> <p>1 A It sounds like it, yes. Habit. It's a</p> <p>2 different definition of habit than I'm used to.</p> <p>3 MS. THOMPSON:</p> <p>4 Q And I think you probably recall when we</p> <p>5 were discussing Health Canada, they were also</p> <p>6 referring to talc, nonasbestiform talc; right?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A I believe so.</p> <p>10 MS. THOMPSON:</p> <p>11 Q And in the -- let's go ahead and mark</p> <p>12 the 2012 IARC that relates to asbestos.</p> <p>13 (DEPOSITION EXHIBIT NUMBER 20</p> <p>14 WAS MARKED FOR IDENTIFICATION.)</p> <p>15 MS. THOMPSON:</p> <p>16 Q That'd be Exhibit 20. And on the first</p> <p>17 page, 219, "The conclusions" -- reading in the</p> <p>18 first paragraph -- "The conclusions reached in</p> <p>19 this monograph about asbestos and its</p> <p>20 carcinogenic risk apply to these six type of</p> <p>21 fibers wherever they are found, and that includes</p> <p>22 talc-containing asbestiform fibers."</p> <p>23 A Yes.</p> <p>24 Q Is that your understanding of this?</p>
<p style="text-align: right;">Page 263</p> <p>1 A Again, I -- I -- that's not in my area</p> <p>2 of expertise.</p> <p>3 MS. THOMPSON:</p> <p>4 Q So you don't know --</p> <p>5 A No.</p> <p>6 Q -- whether there's any difference or</p> <p>7 not?</p> <p>8 A I have no opinion.</p> <p>9 Q And -- well, we can look at the 2010 --</p> <p>10 A Uh-huh.</p> <p>11 Q -- monograph to -- to clarify that.</p> <p>12 So on page 277 --</p> <p>13 A Uh-huh.</p> <p>14 Q -- "Talc may also form" -- reading in</p> <p>15 paragraph 3 --</p> <p>16 A Uh-huh.</p> <p>17 Q -- "Talc may also form as true mineral</p> <p>18 fibers that are asbestiform. Asbestiform</p> <p>19 describes the pattern of growth of a mineral that</p> <p>20 is referred to as a habit."</p> <p>21 And you would agree that that is not</p> <p>22 the same as talc with asbestos; right?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p style="text-align: right;">Page 265</p> <p>1 A I see that. Yeah.</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 MS. THOMPSON:</p> <p>5 Q Would your opinions regarding the</p> <p>6 biological activity of baby powder apply as well</p> <p>7 to baby powder that contains asbestos?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Not asbestiform but asbestos?</p> <p>11 MS. THOMPSON:</p> <p>12 Q Asbestiform, it -- talc with asbestos</p> <p>13 is talc with asbestos.</p> <p>14 A Okay.</p> <p>15 Q Talc with --</p> <p>16 A So it wouldn't change -- it wouldn't</p> <p>17 change my view.</p> <p>18 Q Okay. And what about baby powder that</p> <p>19 contains heavy metals like chromium, nickle, and</p> <p>20 cobalt?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A No.</p> <p>24 MS. THOMPSON:</p>

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<p>1 Q And what about baby powder with</p> <p>2 chemicals that are either possible or known</p> <p>3 carcinogens, like styrene, coumarin, eugenol,</p> <p>4 D'Limonine, p-Cresol, muscutone or benzophenone.</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 MS. THOMPSON:</p> <p>8 Q Would it change your opinion regarding</p> <p>9 the biologic activity of baby powder?</p> <p>10 A Well, looking at the biologic activity</p> <p>11 of baby powder, based upon what I reviewed, the</p> <p>12 answer is no because it doesn't matter what's in</p> <p>13 that. We looked at the biologic activity.</p> <p>14 Q So it doesn't matter to you whether</p> <p>15 there are known carcinogens in baby powder?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A Well, based upon the studies, then we</p> <p>19 would have seen convincing evidence of biologic</p> <p>20 causality. We didn't.</p> <p>21 MS. THOMPSON:</p> <p>22 Q And you're referring to the</p> <p>23 epidemiology studies?</p> <p>24 MS. CURRY:</p>	<p>1 A Okay. Okay. Thank you.</p> <p>2 (DEPOSITION EXHIBIT NUMBER 21 WAS</p> <p>3 MARKED FOR IDENTIFICATION.)</p> <p>4 MS. THOMPSON:</p> <p>5 Q This is Exhibit 21, "Asbestos Exposure</p> <p>6 and Ovarian Fiber Burden."</p> <p>7 Have you seen this paper, Dr. Birrer?</p> <p>8 A So I don't think -- let me -- I don't</p> <p>9 think I reviewed this. Let me just check.</p> <p>10 Well, it was on my list. I must have.</p> <p>11 Q And again, just going to the</p> <p>12 conclusions of these authors, the last paragraph</p> <p>13 in the abstract.</p> <p>14 A Uh-huh.</p> <p>15 Q "This study demonstrates that asbestos</p> <p>16 can reach the ovary. Although the number of</p> <p>17 subjects is small, asbestos appears to be present</p> <p>18 in ovarian tissue more frequently and in higher</p> <p>19 amounts in women with a documentable exposure</p> <p>20 history."</p> <p>21 Do you agree that was the conclusion of</p> <p>22 the authors?</p> <p>23 A That's what they state.</p> <p>24 Q And on page 438, last paragraph, "The</p>
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<p>1 Object to the form.</p> <p>2 A I'm referring to all of it.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Would the presence of known carcinogens</p> <p>5 provide a plausible mechanism?</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A Mechanisms for -- for what?</p> <p>9 MS. THOMPSON:</p> <p>10 Q For possible carcinogenesis.</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A But we didn't see carcinogenesis.</p> <p>14 There's no plausible biologic association or --</p> <p>15 so I'm not sure what we're designing a mechanism</p> <p>16 for.</p> <p>17 MS. THOMPSON:</p> <p>18 Q And are you familiar with the Heller</p> <p>19 paper regarding the finding of asbestos in human</p> <p>20 ovaries?</p> <p>21 A The Heller paper --</p> <p>22 Q 1996?</p> <p>23 A The one we just reviewed or --</p> <p>24 Q I'm handing you a new one.</p>	<p>1 fact that exposure to a husband is more</p> <p>2 significant than exposure to a father suggests a</p> <p>3 possible role for sexual contact as the</p> <p>4 transporting vector for asbestos fibers."</p> <p>5 Would you agree that if sexual -- if</p> <p>6 sexual contact was a transporting vector, that</p> <p>7 the fibers would enter the peritoneal cavity and</p> <p>8 ovaries through the vagina?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A Just ask that once more, please.</p> <p>12 MS. THOMPSON:</p> <p>13 Q That wasn't a very good question. The</p> <p>14 problem is I don't know exactly how to make it</p> <p>15 better.</p> <p>16 If -- if the authors are proposing</p> <p>17 sexual contact as a possible means for</p> <p>18 transporting the asbestos fibers into -- into the</p> <p>19 ovaries, would -- wouldn't you assume that that</p> <p>20 would be via a vaginal route?</p> <p>21 A Yeah, I wouldn't assume that. I think</p> <p>22 one of the challenges here is that there are more</p> <p>23 differences between a wife and a daughter than</p> <p>24 just sexual activity. Wives may be in close</p>

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<p>1 contact with their husband in terms of --</p> <p>2 Q But that's not the question I'm asking.</p> <p>3 I'm saying if sexual contact is a</p> <p>4 transporting vector, wouldn't you assume that</p> <p>5 that would be through a vaginal route, not</p> <p>6 inhalation or some other way?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A If -- if sexual activity was the</p> <p>10 mechanism of transport, is that what you're</p> <p>11 saying?</p> <p>12 MS. THOMPSON:</p> <p>13 Q Right.</p> <p>14 A Yeah.</p> <p>15 It's kind of a non sequitur. I mean,</p> <p>16 you're making the assumption sexual contact, and</p> <p>17 then you're asking, well, if that's it -- if</p> <p>18 that's the mode of transmission, is that the mode</p> <p>19 of transmission. Well, then, you've already</p> <p>20 assumed it, so -- so I could --</p> <p>21 Q Okay. I just wanted to make sure</p> <p>22 you're assuming it because the authors don't</p> <p>23 specifically say, you know, the -- the asbestos</p> <p>24 comes from a perineal exposure --</p>	<p>1 Correct?</p> <p>2 A So it's household contact with men who</p> <p>3 had fairly high exposure. So I think you can</p> <p>4 probably assume it was a substantial amount of</p> <p>5 exposure.</p> <p>6 Q What's your basis for assuming that</p> <p>7 it's a substantial amount of exposure?</p> <p>8 A Well, these men, if they're working in</p> <p>9 the asbestos area, are going to be covered with</p> <p>10 it. That's been shown, which is unfortunate,</p> <p>11 but, yeah.</p> <p>12 Q Can you point me to any study that</p> <p>13 compares how much exposure there would be in a</p> <p>14 talc mine versus a woman using talcum powder on</p> <p>15 her perineum daily or twice daily for -- for</p> <p>16 decades?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A Well, this is not talc. This is not</p> <p>20 talc; this is asbestos.</p> <p>21 MS. THOMPSON:</p> <p>22 Q I know. That's a separate question.</p> <p>23 It's not in the article.</p> <p>24 A Okay. Can you ask that again?</p>
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<p>1 A Well, they're making -- yeah. They're</p> <p>2 making that distinction between a daughter and --</p> <p>3 Q Yeah, they are. I just wanted to make</p> <p>4 sure we are understanding that.</p> <p>5 And in the conclusions, "In our study,</p> <p>6 the women with a positive exposure history had</p> <p>7 asbestos detected in their ovaries more</p> <p>8 frequently and in higher counts."</p> <p>9 If that did indeed happen, that would</p> <p>10 argue against any kind of laboratory</p> <p>11 contamination, wouldn't it?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A I'm just checking the numbers. I'm</p> <p>15 sorry.</p> <p>16 9 of 13 household, 6 of 17 and about</p> <p>17 one out of -- one out of 17.</p> <p>18 So, you know, I think -- I think it's</p> <p>19 fair to say that laboratory contamination should</p> <p>20 be more equal in all groups. It doesn't</p> <p>21 completely eliminate it, but...</p> <p>22 MS. THOMPSON:</p> <p>23 Q And these were exposed through</p> <p>24 household contact, not occupational exposure.</p>	<p>1 Q Can you point me to any study that</p> <p>2 compares how much exposure there would be in a</p> <p>3 talc mine versus a woman using talcum powder on</p> <p>4 her perineum daily or twice daily for decades?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A Yeah. I don't think that's been asked</p> <p>8 and qualified. So it's difficult.</p> <p>9 MS. THOMPSON:</p> <p>10 Q Is the fact that asbestos causes</p> <p>11 pleural and peritoneal mesothelioma relevant to</p> <p>12 whether or not talcum powder can cause ovarian</p> <p>13 cancer?</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 A Not to the data that I -- and the</p> <p>17 studies that I reviewed.</p> <p>18 MS. THOMPSON:</p> <p>19 Q And I don't think this was clear to me</p> <p>20 this morning.</p> <p>21 How does asbestos get to the</p> <p>22 peritoneum, in your opinion?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>

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<p style="text-align: right;">Page 274</p> <p>1 MS. THOMPSON: 2 Q Or do you not know? 3 A Well, I -- I summarized my 4 understanding as not being necessarily an 5 asbestos expert, but my clinical experience, 6 which is asbestos, obviously, is a risk factor 7 for mesothelioma and for lung cancer. If it's 8 inhaled, then it's -- it's transiting to the 9 pleural cavity, which is where, then, it's 10 inducing mesothelioma. 11 And then there are peritoneal 12 mesotheliomas. And I don't honestly think we 13 know precisely how it gets there. There is -- 14 there is some evidence that pleural activities 15 can communicate with peritoneal activities. And 16 the example I'd give you on that is if one has 17 malignant ascites, fluid in the peritoneal 18 cavity, it frequently ends up in the pleural 19 cavities. 20 So -- so -- but you've got diaphragm 21 there with parietal pleura covering it. So 22 exactly how that happens, I don't know. 23 Q Is migration or transport through the 24 genital tract of asbestos a plausible mechanism</p>	<p style="text-align: right;">Page 276</p> <p>1 women who are massively exposed? 2 A I think that's the epidemiologic data 3 I'm aware of. 4 Q You're not aware of the epidemiology 5 that includes household or domestic exposure? 6 MS. CURRY: 7 Object to the form. 8 A Secondary exposures? 9 MS. THOMPSON: 10 Q Correct. 11 A Yeah. Yeah. I know that. I know that 12 a little bit less than the initial occupational 13 exposure. Most -- most of that came from the 14 Army. 15 Q And you'll agree that you don't have 16 any literature that compares what that exposure 17 would be compared to an exposure with someone 18 using talcum powder on their genitals for -- 19 A I agree. 20 Q -- for an extended period of time? 21 A Yes. 22 Q So I want to understand. You don't 23 know whether asbestos fibers can migrate or be 24 transported up the genital tract, but you're</p>
<p style="text-align: right;">Page 275</p> <p>1 for asbestos getting into the peritoneal cavity? 2 MS. CURRY: 3 Object to the form. 4 A Yeah, I don't -- I don't know the 5 answer to that. The increased incidence of 6 ovarian cancer in asbestos-exposed women, I mean, 7 I think it's -- it's agreed upon that those women 8 had massive exposures. So -- 9 MS. THOMPSON: 10 Q What -- what's your basis for saying 11 those women had massive exposures? 12 A Well, my impression is that in gas mask 13 manufacturing -- 14 And, of course, this is in the second 15 world war. 16 -- there wasn't really an appreciation 17 how bad asbestos is. And, so, they got exposed 18 to certainly levels that, you know, average 19 people would not. And even -- even in towns that 20 had cement factories and issues like that, those 21 studies were really not all that positive. But 22 the gas masks are. 23 Q Is it your opinion that the studies 24 that link asbestos with ovarian cancer are all in</p>	<p style="text-align: right;">Page 277</p> <p>1 confident that talc cannot. Is that right? 2 MS. CURRY: 3 Object to the form. 4 A Well, that's part of the reason I don't 5 think asbestos -- we can't say that. If I 6 remember, the question was can -- can the genital 7 tract be an explanation for the asbestos fibers. 8 In my opinion, no, we don't know that. And the 9 data we have from talc suggests, no, that doesn't 10 happen. 11 MS. THOMPSON: 12 Q Still not clear. 13 So asbestos, you don't know; but talc, 14 you know it doesn't. Is that right? 15 MS. CURRY: 16 Object to the form. 17 A Well, I would say, you know, if you -- 18 if you want to pursue that, then I would say, 19 based upon the talc data, which has actually been 20 examined, that it's unlikely that asbestos is 21 going up through the genital tract. 22 MS. THOMPSON: 23 Q So, in your opinion, that is not a 24 plausible mechanism for asbestos reaching the</p>

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<p style="text-align: right;">Page 278</p> <p>1 ovaries?</p> <p>2 A Correct.</p> <p>3 Q And what is your explanation for</p> <p>4 household members of asbestos working -- workers</p> <p>5 having an increased risk of ovarian cancer and</p> <p>6 mesothelioma?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Well, again, not being an asbestos</p> <p>10 expert, but I would assume this is inhalation,</p> <p>11 much like other exposures to asbestos, and then</p> <p>12 absorption through the lung parenchyma and</p> <p>13 ultimately through this pleural perineal process.</p> <p>14 MS. THOMPSON:</p> <p>15 Q But it's your opinion that the transfer</p> <p>16 or migration of the fibers through coitus is not</p> <p>17 plausible?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A I don't know the data for that.</p> <p>21 MS. THOMPSON:</p> <p>22 Q Well, you don't know data for the other</p> <p>23 routes either, do you?</p> <p>24 MS. CURRY:</p>	<p style="text-align: right;">Page 280</p> <p>1 lot more data for -- if it's something to do with</p> <p>2 genital transport than you do for other -- other</p> <p>3 methods, but --</p> <p>4 A Well, I am a scientist.</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 MS. THOMPSON:</p> <p>8 Q Well, it's selective science.</p> <p>9 MS. CURRY:</p> <p>10 Object to the form and argumentative.</p> <p>11 MS. THOMPSON:</p> <p>12 Q If you are advising a patient, could</p> <p>13 you reassure her that talcum powder containing</p> <p>14 asbestos is safe to use on the perineum?</p> <p>15 A It's -- it's an irrelevant issue.</p> <p>16 Q Okay. Patient says, Dr. Birrer, is it</p> <p>17 safe for me to continue using baby powder on the</p> <p>18 per- -- on my perineum. And your answer would</p> <p>19 be?</p> <p>20 A Yes.</p> <p>21 Q And if -- assuming that baby powder</p> <p>22 is -- is shown to contain asbestos, would your</p> <p>23 advice be the same?</p> <p>24 MS. CURRY:</p>
<p style="text-align: right;">Page 279</p> <p>1 Object to the form.</p> <p>2 A Well, there's a lot of literature for,</p> <p>3 you know, shipyard builders where they got</p> <p>4 exposed to asbestos. They get both pleural and</p> <p>5 perineal mesothelioma.</p> <p>6 MS. THOMPSON:</p> <p>7 Q We're talking about household exposure.</p> <p>8 A But again, that's data to tell us,</p> <p>9 under the extreme conditions, where and how that</p> <p>10 might migrate.</p> <p>11 Q Well, but you don't believe Heller, who</p> <p>12 proposed that sexual transmission was a plausible</p> <p>13 route for -- for the asbestos fibers in contacts</p> <p>14 to have a higher incidence of ovarian cancer in</p> <p>15 perineal mesothelioma; right?</p> <p>16 MS. CURRY:</p> <p>17 Object to the form.</p> <p>18 A Well, they didn't say that. They</p> <p>19 didn't say that. They said it's possible.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Okay.</p> <p>22 A They're proposing a hypothesis and I</p> <p>23 said, well, show me the data.</p> <p>24 Q Okay. Well, it seems like you need a</p>	<p style="text-align: right;">Page 281</p> <p>1 Object to the form.</p> <p>2 MS. THOMPSON:</p> <p>3 Q Would your answer be the same?</p> <p>4 A So this is a hypothetical?</p> <p>5 Q Yeah.</p> <p>6 A Powder is the -- is -- is then</p> <p>7 determined to have asbestos?</p> <p>8 Q Correct.</p> <p>9 A Again, so is the question am I</p> <p>10 recommending a patient use asbestos?</p> <p>11 Q Yeah. That's the question.</p> <p>12 A Yeah. No, I wouldn't do that.</p> <p>13 Q Did you read Dr. Longo's report?</p> <p>14 A You know, that came up.</p> <p>15 Can you -- do you have a copy of it to</p> <p>16 refresh my memory?</p> <p>17 Q I do.</p> <p>18 (DEPOSITION EXHIBIT NUMBER 22 WAS</p> <p>19 MARKED FOR IDENTIFICATION.)</p> <p>20 MS. THOMPSON:</p> <p>21 Q I'm gonna mark -- Exhibit 22 is</p> <p>22 Dr. Longo's report in the MDL.</p> <p>23 Exhibit 23 is Dr. Longo's supplemental</p> <p>24 report in the MDL.</p>

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<p>1 (DEPOSITION EXHIBIT NUMBER 23 WAS 2 MARKED FOR IDENTIFICATION.) 3 MS. THOMPSON: 4 Q Do you remember seeing these reports? 5 MS. CURRY: 6 Do you have an extra copy? 7 MS. THOMPSON: 8 I do. 9 MS. CURRY: 10 Thank you. 11 A It's not on my list. 12 MS. THOMPSON: 13 Q Did you ask to see any testing on 14 Johnson's baby powder to see if it contained 15 asbestos? 16 A No, I did not. I think I came across 17 this, actually, previously, but not in this one. 18 Q And understanding that you're -- well, 19 I assume that you're not an expert in asbestos 20 testing; right? 21 A Correct. 22 Q Assuming that -- and if you want to 23 read the report, we can go off the record. 24 But assuming that Dr. Longo found</p>	<p>1 telling a patient it was safe to use baby powder 2 on her genitals if it contained -- if two-thirds 3 of the bottles contained asbestos? 4 MS. CURRY: 5 Object to the form. 6 A You know, again, I'm gonna emphasize 7 this. My review of the data suggests that -- 8 that those products are not a risk for ovarian 9 cancer. 10 MS. THOMPSON: 11 Q I -- I'm clear -- 12 A Regardless of what the hypothetical is. 13 Q I'm clear on that. 14 A Okay. 15 Q But -- but this is not really even a 16 hypothetical. This is testing that has shown 17 two-thirds of the baby powder samples contain 18 asbestos. 19 Do -- would you still feel good about 20 advising a patient that it's safe? 21 MS. CURRY: 22 Object to the form. 23 A I would -- I would tell them that based 24 on my review of the literature, extensive review</p>
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<p>1 between 60 and 70 percent of bottles, historical 2 samples provided by Johnson & Johnson over 3 decades to contain asbestos, would that impact 4 how you would advise a patient who says, 5 Dr. Birrer, is it safe for me to use Johnson's 6 baby powder on my perineum? 7 MS. CURRY: 8 Object to the form. 9 A So, again, this -- this gets to the 10 point of having reviewed all the literature in 11 terms of the product, Shower to Shower, 12 Johnson & Johnson's baby powder, as increasing 13 the risk for ovarian cancer showing biological 14 plausibility. 15 Careful review of that literature has 16 shown nothing. So whether there's asbestos in 17 there or not, I don't know. 18 MS. THOMPSON: 19 Q Would -- would it give you pause? 20 MS. CURRY: 21 Object to the form. 22 A Pause. I don't know what pause is. 23 MS. THOMPSON: 24 Q Would you have some concern about</p>	<p>1 of the literature, it is a safe product. 2 MS. THOMPSON: 3 Q And what if they said, Dr. Birrer, is 4 that true even if it does contain asbestos? 5 MS. CURRY: 6 Object to the form. 7 MS. THOMPSON: 8 Q Would your answer be the same? 9 A I would -- I would -- you know, I would 10 say, again, it doesn't matter if that's the way 11 the product was used. And it was careful 12 studies. 13 Q Have you seen any studies from 14 Johnson & Johnson regarding their asbestos 15 testing? 16 A I haven't seen that. 17 Q Were you shown any testing results from 18 Johnson & Johnson? 19 A No. 20 Q Were you shown any testing results from 21 defense experts as to whether baby powder 22 contained asbestos? 23 MS. CURRY: 24 Object to the form.</p>

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<p style="text-align: right;">Page 286</p> <p>1 A Not that I recall, although, as I said</p> <p>2 before, in the expert witness reports, the ones</p> <p>3 that involved minerals in asbestos, I went</p> <p>4 through them fairly rapidly.</p> <p>5 MS. THOMPSON:</p> <p>6 Q Do you know if any defense experts even</p> <p>7 performed any testing as to whether there was</p> <p>8 asbestos in baby powder?</p> <p>9 A No.</p> <p>10 Q Do you know -- did you see that</p> <p>11 Dr. Longo also tested for talc fibers, so-called</p> <p>12 fibrous talc?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A Fibrous talc. I can't quote you that,</p> <p>16 but I'll rely on you.</p> <p>17 MS. THOMPSON:</p> <p>18 Q Dr. Longo found -- and, you know, feel</p> <p>19 free to look to that summary -- virtually every</p> <p>20 Johnson's baby powder and Shower to Shower sample</p> <p>21 provided from historical samples contained talc</p> <p>22 fibers. The same answer as to asbestos; it</p> <p>23 doesn't matter?</p> <p>24 MS. CURRY:</p>	<p style="text-align: right;">Page 288</p> <p>1 A No, I didn't. I see the litigation ad.</p> <p>2 MS. THOMPSON:</p> <p>3 Q Okay. I'm gonna give you -- I'm gonna</p> <p>4 mark as Exhibit 24 a report -- call it an article</p> <p>5 because it's titled "News" -- from BMJ. And</p> <p>6 what's BMJ?</p> <p>7 A I don't know. I was gonna ask you.</p> <p>8 Q Oh. British Medical Journal. You've</p> <p>9 heard of the British Medical Journal?</p> <p>10 A Yes. I thought it was Birmingham.</p> <p>11 Q I -- that was another trick question.</p> <p>12 I said it was a news report from a medical</p> <p>13 journal.</p> <p>14 And you can take a minute to look</p> <p>15 through that --</p> <p>16 A Please.</p> <p>17 Q -- since you haven't seen the news</p> <p>18 reports.</p> <p>19 So you'll, I think, agree with me that</p> <p>20 the editors didn't come to any conclusions as to</p> <p>21 whether or not baby powder caused ovarian cancer;</p> <p>22 right?</p> <p>23 A Correct.</p> <p>24 Q But they -- the editors of the journal</p>
<p style="text-align: right;">Page 287</p> <p>1 Object to the form.</p> <p>2 A There again, these products that he's</p> <p>3 analyzing have been used for years. We have the</p> <p>4 epi data. It's unconvincing. We've got the</p> <p>5 biologic data. It's definitely unconvincing.</p> <p>6 The inflammatory theory is inconsistent. So to</p> <p>7 say anything other than that this is a safe</p> <p>8 product, I think, is inappropriate.</p> <p>9 MS. THOMPSON:</p> <p>10 Q Are -- are you aware of news reports</p> <p>11 over the past two or three months of the presence</p> <p>12 of asbestos in baby powder and</p> <p>13 Johnson & Johnson's knowledge of the asbestos in</p> <p>14 baby powder?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A I'm not.</p> <p>18 (DEPOSITION EXHIBIT NUMBER 24</p> <p>19 WAS MARKED FOR IDENTIFICATION.)</p> <p>20 MS. THOMPSON:</p> <p>21 Q You haven't seen any news reports about</p> <p>22 asbestos in baby powder?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>	<p style="text-align: right;">Page 289</p> <p>1 at least thought it important to -- to report the</p> <p>2 claims that baby powder may contain asbestos;</p> <p>3 correct?</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 A I think they thought this would be of</p> <p>7 interest to the readership.</p> <p>8 MS. THOMPSON:</p> <p>9 Q Agreed.</p> <p>10 And you don't think the editors would</p> <p>11 have published this news report if it wasn't</p> <p>12 based on what they considered credible evidence,</p> <p>13 would you?</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 A I would -- I would not agree with that</p> <p>17 statement. I think they would -- they might not</p> <p>18 agree with any of this or the role of talcum</p> <p>19 powder or asbestos, but -- but they felt their</p> <p>20 readership would be interested in this.</p> <p>21 MS. THOMPSON:</p> <p>22 Q So BMJ has become the National Enquirer</p> <p>23 of medical journals now?</p> <p>24 MS. CURRY:</p>

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<p>1 Object to the form.</p> <p>2 A Medical journals are not above some</p> <p>3 editorial latitude.</p> <p>4 MS. THOMPSON:</p> <p>5 Q And why would the readers be</p> <p>6 interested?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Well, I think there -- there is major</p> <p>10 litigation involved. There are a number of court</p> <p>11 cases. The FDA has weighed in a little bit. And</p> <p>12 then there are, quote, internal documents. All</p> <p>13 of that is, for lack of a better word, you know,</p> <p>14 scientists are looking for things to excite their</p> <p>15 lives, so this is entertainment.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Might it be that BMJ thought their</p> <p>18 doctors would want to tell patients about this</p> <p>19 information?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 MR. MIZGALA:</p> <p>23 So now you're --</p> <p>24 MS. THOMPSON:</p>	<p>1 conclusions. You're a physician and you see this</p> <p>2 article. Might it be something that you would be</p> <p>3 interested in so you could advise your patients</p> <p>4 accordingly?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A Definitely not.</p> <p>8 MS. THOMPSON:</p> <p>9 Q And you would not give a medical</p> <p>10 journal any credit that doctors might want to</p> <p>11 advise their patients that baby powder contains</p> <p>12 asbestos?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A I think they do a reasonable job of</p> <p>16 simply reporting what is happening. And they</p> <p>17 talk about -- they talk about internal documents.</p> <p>18 Those are essentially impossible to assess. They</p> <p>19 talk about the New York Times. Not a scientific</p> <p>20 organization. There is some hearsay from the</p> <p>21 FDA. And then they -- they out line the court</p> <p>22 case. I wouldn't -- I would not take this and</p> <p>23 translate it into some recommendation for a</p> <p>24 patient.</p>
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<p>1 Q Just a hunch. Just a hunch.</p> <p>2 MR. MIZGALA:</p> <p>3 Now you're asking him to speculate.</p> <p>4 You've been doing this the whole deposition.</p> <p>5 MS. GARBER:</p> <p>6 I don't think we're doing speaking</p> <p>7 objections. So the objection is to form.</p> <p>8 MR. MIZGALA:</p> <p>9 Yeah. But she's gone to task for</p> <p>10 speculating earlier, and she's doing the same</p> <p>11 thing.</p> <p>12 MS. GARBER:</p> <p>13 Okay. The objection is to form. You</p> <p>14 know that. Let's follow the rules.</p> <p>15 A Say again.</p> <p>16 MS. THOMPSON:</p> <p>17 Q You're a physician that reads journals.</p> <p>18 A Uh-huh.</p> <p>19 Q As a physician, let's -- we're going to</p> <p>20 take a hypothetical that you're not involved in</p> <p>21 talcum powder litigation. Okay?</p> <p>22 A Uh-huh.</p> <p>23 Q And you haven't done this thorough</p> <p>24 review that you have done to come to your</p>	<p>1 MS. THOMPSON:</p> <p>2 Q So it wouldn't be any different from</p> <p>3 reading a story about the Kardashians in BMJ?</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 MS. THOMPSON:</p> <p>7 Q Is that what you're saying?</p> <p>8 A You want an answer to that?</p> <p>9 Q Sure. It was a question.</p> <p>10 A Yeah, it's different.</p> <p>11 Q Okay. Thanks.</p> <p>12 A It's about talc.</p> <p>13 Q Are you aware that concerns have been</p> <p>14 raised about the safety of pleurodesis?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A So, actually, my understanding of</p> <p>18 pleurodesis, at least in the relationship of talc</p> <p>19 in ovarian cancer, there's essentially no</p> <p>20 evidence linking the two. But let me -- let me</p> <p>21 see what you're referring to.</p> <p>22 MS. THOMPSON:</p> <p>23 Q Well, I was just -- let me ask</p> <p>24 questions first.</p>

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<p>1 A Uh-huh.</p> <p>2 Q And that was: Are you aware that</p> <p>3 concerns have been raised about the safety of</p> <p>4 pleurodesis?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A No.</p> <p>8 MS. THOMPSON:</p> <p>9 Q And have you been -- are you aware --</p> <p>10 no, you're not aware of any concerns at all.</p> <p>11 Let me go ahead and give you Exhibit</p> <p>12 25.</p> <p>13 (DEPOSITION EXHIBIT NUMBER 25</p> <p>14 WAS MARKED FOR IDENTIFICATION.)</p> <p>15 MS. THOMPSON:</p> <p>16 Q And this is a letter to the editor.</p> <p>17 I --</p> <p>18 A Uh-huh.</p> <p>19 Q -- I understand that. It's not a</p> <p>20 formal study, per se.</p> <p>21 MS. CURRY:</p> <p>22 Do you have an extra copy?</p> <p>23 MS. THOMPSON:</p> <p>24 Yeah, I do.</p>	<p>1 stating that talc is asbestos-free should not</p> <p>2 release us from a responsibility to the patient,</p> <p>3 especially when safe alternatives are available."</p> <p>4 And the picture is of a talc fiber</p> <p>5 found in a pleurodesis talc.</p> <p>6 Does that cause you any concern?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A It doesn't. To be fair, the entire --</p> <p>10 my -- my impression is, although I don't do -- I</p> <p>11 do pleurodesis for cancer patients, in which</p> <p>12 case, unfortunately, longevity makes this whole</p> <p>13 issue moot. But we've moved away from talc for</p> <p>14 other reasons. It's painful. It doesn't work</p> <p>15 all the time. We have better agents. So that</p> <p>16 kind of makes this moot.</p> <p>17 But, you know, again I think you</p> <p>18 pointed out appropriately. It's -- they're</p> <p>19 entitled to their opinions. It's a single</p> <p>20 article -- it's a single letter, and the studies</p> <p>21 addressing this are very limited. So I think --</p> <p>22 I think they're -- making fairly bold statements</p> <p>23 on not a lot of data.</p> <p>24 MS. THOMPSON:</p>
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<p>1 Q Do you know Dr. -- I think it's Ghio.</p> <p>2 I don't know how it's pronounced. Do you know</p> <p>3 Ghio and Dr. Roggli?</p> <p>4 A I don't know either of them.</p> <p>5 Q And I'll let you read through this.</p> <p>6 Let's just read that -- I'm gonna read the last</p> <p>7 paragraph and get your thoughts.</p> <p>8 A Okay.</p> <p>9 Q "The assertion that contemporary</p> <p>10 purified preparations of talc do not contain</p> <p>11 asbestos, therefore eliminating a risk of</p> <p>12 mesothelioma, should be closely examined prior to</p> <p>13 its acceptance for clinical application. The</p> <p>14 methodology used to confirm the lack of</p> <p>15 asbestiform materials in a finished product,</p> <p>16 (i.e., X-ray diffraction, optical microscopy, and</p> <p>17 electron microscopy techniques) and its</p> <p>18 sensitivity must be provided. Even if the</p> <p>19 product is "asbestos-free," the mechanism of</p> <p>20 cancer induction by asbestos (i.e.,</p> <p>21 metal-catalyzed radical generation) is similarly</p> <p>22 pertinent to talc and the occurrence of fibrous</p> <p>23 forms of the sheet silicate itself raises issues</p> <p>24 about clearance and long-term safety. Simply</p>	<p>1 Q But you'll agree that this was out of</p> <p>2 the context of any litigation about baby powder;</p> <p>3 correct?</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 A I would agree on that.</p> <p>7 MS. THOMPSON:</p> <p>8 Q What's your understanding of the</p> <p>9 mechanism by which asbestos causes cancer?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A Again, I'm not necessarily an expert on</p> <p>13 this. The association and the risk factor's very</p> <p>14 clear. I think the present theory -- and I would</p> <p>15 put it as a theory -- is this is a substance that</p> <p>16 essentially doesn't dissolve, stays there, or at</p> <p>17 least is very long-lasting, and then, under those</p> <p>18 circumstances, causes effectively the</p> <p>19 transformation of cells that it is in close</p> <p>20 contact with. And that's -- it includes, of</p> <p>21 course, lung cancer per se, but also mesothelioma</p> <p>22 where these particles will sort of stay in the</p> <p>23 pleural cavity.</p> <p>24 MS. THOMPSON:</p>

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<p style="text-align: right;">Page 298</p> <p>1 Q Is there anything in that description 2 that you gave that would be different for talc? 3 MS. CURRY: 4 Object to the form. 5 A Well -- 6 MS. THOMPSON: 7 Q And we're speaking in general terms. 8 MS. CURRY: 9 Object to the form. 10 A Talc doesn't do this; right? 11 MS. THOMPSON: 12 Q Well, no. Let's go back. 13 You would agree that talc essentially 14 doesn't dissolve also; correct? 15 MS. CURRY: 16 Object to the form. 17 A It's a mineral. 18 MS. THOMPSON: 19 Q And it stays there; correct? 20 MS. CURRY: 21 Object to the form. 22 A Well, I don't know if it stays there as 23 long as asbestos. You know, if you look at the 24 pleurodesis patients, there's really essentially</p>	<p style="text-align: right;">Page 300</p> <p>1 because I wasn't asked to review that, and -- and 2 my experience is in lung cancer. 3 That process, I think, is still -- is 4 still questionable. And -- and because of that, 5 that -- that process may be specifically 6 associated with asbestos. So to extrapolate that 7 to some other molecule that, oh, by the way, it 8 hangs around for a while, is not acceptable. 9 Q So I understand that you apparently 10 were not asked to consider asbestos. You're a 11 scientist; right? 12 A Yes. 13 Q Did you not have any curiosity about 14 what effects the presence of asbestos in baby 15 powder would have? 16 MS. CURRY: 17 Object to the form. 18 A To be honest, that wasn't the way I 19 approached it. I approached it by looking 20 specifically from the talc standpoint. 21 MS. THOMPSON: 22 Q Okay. 23 A And -- and the studies and then looking 24 at that objectively. And, again, we get back to</p>
<p style="text-align: right;">Page 299</p> <p>1 no increase in ovarian cancer. 2 MS. THOMPSON: 3 Q Well, you've already told us that 4 pleurodesis patients have typically a life 5 expectancy of months, not years. 6 MS. CURRY: 7 Object to the form. 8 A I said in the ones I treat. But in 9 chronic heart failure, those patients have been 10 followed up to 40 years. 11 MS. THOMPSON: 12 Q I would like to see that study, but 13 we'll do that another day. How's that? 14 A I don't know if I'd like another day. 15 Q Let's say -- or -- your next comment, 16 or at least it's very long-lasting. You would 17 agree that -- with that for talc; right? 18 A Uh-huh. Uh-huh. 19 Q And, then, for asbestos, you say it 20 causes effectively the transformation of cells 21 that it's in close contact with. But you don't 22 believe that happens for talc; correct? 23 A Well, again, this may reflect my -- 24 somewhat my ignorance about asbestos per se,</p>	<p style="text-align: right;">Page 301</p> <p>1 this issue of really looking at epidemiologic 2 studies, just use powder, and then some of the 3 studies biologically used it -- use those -- used 4 those products. It -- you know, if there are -- 5 if there are substance X, Y, Z, A, B, and C that 6 are in there that are causing a problem and 7 carcinogenic, it would have shown up in the 8 studies. 9 Q Do you know that initially in the 10 studies, asbestos, no one could prove that 11 asbestos was carcinogenic? 12 MS. CURRY: 13 Object to the form. 14 A Well, no one could prove smoking was 15 carcinogenic either. It takes time. 16 MS. THOMPSON: 17 Q Well, there's two examples then. 18 (DEPOSITION EXHIBIT NUMBER 26 19 WAS MARKED FOR IDENTIFICATION.) 20 MS. THOMPSON: 21 Q I'm going to show you Exhibit 26, a 22 paper by Dr. Mossman. Do you know Mossman? 23 A I do know Dr. Mossman. Not personally. 24 Q You know her by reputation?</p>

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<p style="text-align: right;">Page 302</p> <p>1 A I think we shared classmates about 20</p> <p>2 years ago.</p> <p>3 Q I -- I won't -- I won't go any further</p> <p>4 with that one.</p> <p>5 The title of this study is "Mechanistic</p> <p>6 in vitro studies: What they have told us about</p> <p>7 carcinogenic properties of elongated mineral</p> <p>8 particles."</p> <p>9 I think we've already established that</p> <p>10 that's not a term that you're particularly</p> <p>11 familiar with. But go ahead and take a minute to</p> <p>12 look at --</p> <p>13 A 26?</p> <p>14 Q -- that paper.</p> <p>15 And I'm going to just read from the</p> <p>16 abstract. "In vitro studies using target and</p> <p>17 effector cells of mineral-induced cancers have</p> <p>18 been critical in determining the mechanisms of</p> <p>19 pathogenesis as well as the properties" --</p> <p>20 A Where are you?</p> <p>21 Q The first sentence of the paper, in the</p> <p>22 abstract.</p> <p>23 A Oh, okay. Thank you.</p> <p>24 Q "In vitro studies" -- we'll start over.</p>	<p style="text-align: right;">Page 304</p> <p>1 Object to the form.</p> <p>2 MS. THOMPSON:</p> <p>3 Q That in vitro studies could be used to</p> <p>4 test that mechanism in EMPs?</p> <p>5 A And she's --</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A -- she's well respected in this area.</p> <p>9 MS. THOMPSON:</p> <p>10 Q We're going to get to Saed's, Dr.</p> <p>11 Saed's work in a minute.</p> <p>12 A Okay.</p> <p>13 Q But wouldn't you agree that that's what</p> <p>14 Dr. Saed started testing in his in vitro studies?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A I think the expert report and the paper</p> <p>18 that I read is within this spectrum.</p> <p>19 MS. THOMPSON:</p> <p>20 Q And, just moving down a little bit,</p> <p>21 maybe two-thirds of the way down, "Comparative</p> <p>22 studies using chemical carcinogens showed that</p> <p>23 chemical agents interacted directly with DNA;</p> <p>24 whereas, long EMPs appeared to be promoters of</p>
<p style="text-align: right;">Page 303</p> <p>1 "In vitro studies using target and</p> <p>2 effector cells of mineral-induced cancers have</p> <p>3 been critical in determining the mechanisms of</p> <p>4 pathogenesis as well as the properties of</p> <p>5 elongated mineral particles, EMPs, important in</p> <p>6 eliciting these responses."</p> <p>7 Dr. Mossman is reporting that in vitro</p> <p>8 studies have been helpful in -- in determining</p> <p>9 this mechanism; right?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A Yeah, I think that's what she's saying.</p> <p>13 Yes.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Next sentence, "Historically, in vitro</p> <p>16 models of mutagenesis and immortalized cell lines</p> <p>17 were first used to test the theory that EMPs were</p> <p>18 mutagenic to cells, and genotoxicity, as defined</p> <p>19 as damage to DNA, often culminating in cell</p> <p>20 death, was observed in a dose-dependent fashion</p> <p>21 as responses of many cell types to a number of</p> <p>22 EMPs."</p> <p>23 Does that sound reasonable?</p> <p>24 MS. CURRY:</p>	<p style="text-align: right;">Page 305</p> <p>1 cancer via a number of mechanisms, such as</p> <p>2 inflammation, generation of oxidants and</p> <p>3 instigation of cell division.</p> <p>4 "The multitude of these signaling</p> <p>5 cascades and epigenetic mechanisms of both lung</p> <p>6 cancers and mesotheliomas have been most recently</p> <p>7 studied in normal or telomerase immortalized</p> <p>8 human cells."</p> <p>9 I believe she's saying -- and I'll ask</p> <p>10 you if it's correct -- that particles,</p> <p>11 particularly the elongated particles or fibers,</p> <p>12 have a different mechanism than what is usually</p> <p>13 thought of with chemical carcinogens.</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Is that a --</p> <p>18 A I think that's --</p> <p>19 Q -- reasonable interpretation?</p> <p>20 A You know, again, we've been down this</p> <p>21 road a little bit. This is a review article, so</p> <p>22 she's kind of looking at it globally. But I</p> <p>23 think that what you describe is one of the, sort</p> <p>24 of, take-home messages she's implying.</p>

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<p style="text-align: right;">Page 306</p> <p>1 Q Thank you. I'm honored --</p> <p>2 A Okay. We're done?</p> <p>3 Q -- to have kind of gotten it right.</p> <p>4 A We're done?</p> <p>5 Q No.</p> <p>6 A No?</p> <p>7 Q But I'm gonna shave 10 minutes off for</p> <p>8 that compliment.</p> <p>9 And in the paragraph 2, "General</p> <p>10 Concepts of Cancer Development," first</p> <p>11 paragraph --</p> <p>12 MS. CURRY:</p> <p>13 I'm sorry. The realtime is not --</p> <p>14 (Off the record.)</p> <p>15 A I wouldn't -- we -- can we sort of edge</p> <p>16 towards a break at some point?</p> <p>17 MS. THOMPSON:</p> <p>18 Q Yeah. Let's just go ahead and just</p> <p>19 finish -- almost finished, and then we'll come</p> <p>20 back. That's a good -- good spot.</p> <p>21 (Technical difficulties with realtime.)</p> <p>22 MS. THOMPSON:</p> <p>23 Q Are we okay going forward for a couple</p> <p>24 questions without the realtime?</p>	<p style="text-align: right;">Page 308</p> <p>1 MS. THOMPSON:</p> <p>2 Q Would you agree that some scientists</p> <p>3 tend to like one explanation or the other, and</p> <p>4 the other scientists liking a different</p> <p>5 explanation more than the first one?</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A I think that -- I think if you look at</p> <p>9 the investigators in this field, they'll come at</p> <p>10 it, as their expertise, from one direction or the</p> <p>11 other.</p> <p>12 But, you know -- you know, Brook is</p> <p>13 somebody who sees the big picture. I'd like to</p> <p>14 think I do, too. So there's some of us who look</p> <p>15 at the whole thing.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Okay. That's a good explanation.</p> <p>18 But there are scientists doing credible</p> <p>19 work that are kind of in both camps?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A I think that's fair.</p> <p>23 MS. THOMPSON:</p> <p>24 Q And then I'm going to that next page.</p>
<p style="text-align: right;">Page 307</p> <p>1 A Yes.</p> <p>2 Q So in number 2, "General Concepts of</p> <p>3 Cancer Development."</p> <p>4 A Uh-huh.</p> <p>5 Q "The development and use of in vitro</p> <p>6 models over time has corresponded with the</p> <p>7 evolution of research and knowledge on cancer</p> <p>8 etiology in humans."</p> <p>9 Would you agree with that statement?</p> <p>10 A I think so, yes.</p> <p>11 Q Next sentence, "While some scientists</p> <p>12 have suggested that the relative contributions of</p> <p>13 DNA replications and mutations are overwhelming</p> <p>14 drivers of cancer risk, others argue that</p> <p>15 experimental and evolutionary data point to</p> <p>16 tissue microenvironment and epigenetic changes as</p> <p>17 being key to tumorigenesis."</p> <p>18 Would you agree with that statement?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A I think it's a quantitative issue. So</p> <p>22 in some tumors, mutagenesis takes prominence; in</p> <p>23 others, the microenvironment is important. And</p> <p>24 it's a spectrum.</p>	<p style="text-align: right;">Page 309</p> <p>1 I just have, I think, one more passage I'd like</p> <p>2 to read from this paper and get -- get your</p> <p>3 thoughts.</p> <p>4 The first full paragraph on the second</p> <p>5 page of the article, page 63, "The modern day</p> <p>6 definition of epigenetic mechanisms has evolved</p> <p>7 over time to encompass the fact that alterations</p> <p>8 in the primary structure of DNA do not underlie</p> <p>9 most changes in the development of tumors.</p> <p>10 Accordingly, an epigenetic trait can be a stable</p> <p>11 inheritable phenotype resulting from changes in a</p> <p>12 chromosome without alteration in the DNA</p> <p>13 sequence."</p> <p>14 Do you agree with that statement?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A It strikes me as a little overstated,</p> <p>18 particularly the first part, "...epigenetic</p> <p>19 mechanism evolved over time to encompass the fact</p> <p>20 that alterations in the primary structure do not</p> <p>21 underline most changes." That, I -- I'm not sure</p> <p>22 where that's coming from.</p> <p>23 Now, it may be in a single tumor,</p> <p>24 epigenetic is more important than mutation; but</p>

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<p style="text-align: right;">Page 310</p> <p>1 in others, a mutation would be more important. 2 Again, when we treat patients, as you 3 know, we're sequencing everything, and that's not 4 looking at epigenetics. It's looking at 5 mutations. Tumors are riddled with these things. 6 In fact, the problem that we face is what's the 7 driver versus the passenger. 8 MS. THOMPSON: 9 Q So in a particular tumor, either 10 mechanism -- well, it could be either mechanism 11 or both in various amount of contribution. Is 12 that a fair statement? 13 MS. CURRY: 14 Object to the form. 15 A I think it's a fair statement. 16 MS. THOMPSON: 17 Let's take a break. 18 VIDEOGRAPHER: 19 Off the record at 3:26 p.m. 20 (OFF THE RECORD.) 21 VIDEOGRAPHER: 22 We're back on the record at 3:45 p.m. 23 MS. THOMPSON: 24 Q Dr. Birrer, let's talk about Dr. Saed</p>	<p style="text-align: right;">Page 312</p> <p>1 So -- and then he did a fair amount of work on 2 adhesion, pure adhesion. 3 MS. THOMPSON: 4 Q And his adhesion work involved 5 oxidative stress in adhesions, didn't it? 6 A I think he would argue that. I 7 didn't -- it wasn't clear to me from my 8 perspective. But that's a component of what he 9 looked at. The unifying factor for me is that 10 it's gynecologic. 11 Q Okay. 12 A Okay. 13 Q And he has 234 peer-reviewed 14 publications; correct? Oh, no. Take that back. 15 A 136, isn't it? 16 Q 136. I was looking -- 17 A 136. Correct. 18 Q What is oxidative stress? 19 A Well, that's -- that's a biochemical 20 state, if you will, within -- we -- we consider 21 as biologists within cells. It exists in all 22 cells. And it's a balance between ox- -- you 23 know, oxidizing effects and antioxidants. 24 As a term, oxidative, of course, it's a</p>
<p style="text-align: right;">Page 311</p> <p>1 and his research. Okay? 2 A Okay. 3 Q Did you look at Dr. Saed's CV? 4 A I did. 5 Q I'll go ahead and mark that as exhibit 6 27. 7 (DEPOSITION EXHIBIT NUMBER 27 WAS 8 MARKED FOR IDENTIFICATION.) 9 A Thank you. 10 MS. THOMPSON: 11 Q And looking at his CV, would you agree 12 that the focus of his lab has been the study of 13 oxidative stress and its biological effects? 14 MS. CURRY: 15 Object to the form. 16 A Let me refresh my -- refresh my memory 17 on this a little bit. 18 So I think, you know, looking at, if I 19 recall correctly -- I would say that he -- one of 20 his -- one of the components of what he looks at 21 is oxidative stress. If you look at his career, 22 he's been fairly broadly over a broad number of 23 topics. He's looked at, like, gene amplification 24 in certain tumors, mostly in GYN, I might add.</p>	<p style="text-align: right;">Page 313</p> <p>1 chemistry definition. But this one, I think what 2 he means by oxidative stress is it's -- or what 3 you're implying is it's a biologic process. 4 Okay? 5 Q And is it fair to say that at least 6 some scientists believe that oxidative stress 7 plays a role in the etiology of many types of 8 cancers? 9 MS. CURRY: 10 Object to the form. 11 A I think it's safe to say oxidative 12 stress has been investigated and associated with 13 some cancers. 14 MS. THOMPSON: 15 Q Okay. Do you have an opinion on the 16 role of oxidative stress in the initiation of 17 ovarian cancer? 18 A I think that's unresolved at this 19 point. Most of the data that I know of for 20 oxidative stress, a lot of the data is in ovarian 21 tumors. They're already established. 22 Q Are -- would you say there are 23 scientists on both sides of that issue? 24 MS. CURRY:</p>

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<p>1 Object to the form.</p> <p>2 A Would you define that, please?</p> <p>3 MS. THOMPSON:</p> <p>4 Q The importance of oxidative stress in</p> <p>5 the pathogenesis of ovarian cancer.</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A I think it's an area of active</p> <p>9 investigation.</p> <p>10 MS. THOMPSON:</p> <p>11 Q Okay. So you would agree that</p> <p>12 researchers who believe that oxidative stress</p> <p>13 plays a role in the initiation or progression of</p> <p>14 ovarian cancer are not unreasonable?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A It's a generalization that I can't</p> <p>18 comment on. Which researchers?</p> <p>19 MS. THOMPSON:</p> <p>20 Q Okay. But they wouldn't automatically</p> <p>21 be unreasonable?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A Because they believe --</p>	<p>1 A Yeah.</p> <p>2 Q Let's go to your report.</p> <p>3 A We're done with the CV?</p> <p>4 Q I think so.</p> <p>5 A Are you going to the report or the</p> <p>6 paper?</p> <p>7 Q I'm going to your report first.</p> <p>8 A Yeah. Okay.</p> <p>9 Q And then the report, I'll probably go</p> <p>10 to the -- this paper next.</p> <p>11 So in your report, going to page --</p> <p>12 actually, let's start on page 19.</p> <p>13 A Uh-huh.</p> <p>14 Q And you have the big heading, Section</p> <p>15 4 --</p> <p>16 A Uh-huh.</p> <p>17 Q -- Dr. Saed's Plaintiff-Funded</p> <p>18 Research.</p> <p>19 Did you write that heading?</p> <p>20 A Yes.</p> <p>21 Q What is the basis for calling</p> <p>22 Dr. Saed's research plaintiff-funded?</p> <p>23 A My understanding is after he submitted</p> <p>24 his -- the preprint said -- revealed,</p>
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<p>1 MS. THOMPSON:</p> <p>2 Q Because they believe in the importance</p> <p>3 of oxidative stress.</p> <p>4 A I don't think so.</p> <p>5 Q They wouldn't automatically be</p> <p>6 credible -- not credible?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A That would depend on the work they've</p> <p>10 done --</p> <p>11 MS. THOMPSON:</p> <p>12 Q Okay.</p> <p>13 A -- in their experiments.</p> <p>14 Q All right. And they wouldn't</p> <p>15 automatically be uninformed. Would you agree</p> <p>16 with that?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 MS. THOMPSON:</p> <p>20 Q It would depend?</p> <p>21 A We need to look at their -- their</p> <p>22 scientific investigation to determine if they're</p> <p>23 uninformed.</p> <p>24 Q Okay.</p>	<p>1 essentially, nothing, and then the actual paper,</p> <p>2 I believe, said that he was -- that he was a</p> <p>3 consultant and an expert witness.</p> <p>4 Q Does that mean to you plaintiff-funded</p> <p>5 research?</p> <p>6 A Well, I mean, that was a separate</p> <p>7 issue, that there was money actually flowing into</p> <p>8 his lab.</p> <p>9 Q What -- what is your basis for saying</p> <p>10 there was money flowing into his lab?</p> <p>11 A I think that's what we -- I saw in</p> <p>12 his -- let me see. Hang on -- his deposition.</p> <p>13 Q What did his deposition say about that?</p> <p>14 A I'd have to refresh my memory. Do you</p> <p>15 have it?</p> <p>16 Q Do you recall that the funding for the</p> <p>17 research came from his university lab funds and</p> <p>18 that he was paid for his time as a consultant?</p> <p>19 Does that sound right?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A I think I remember that the exchange</p> <p>23 was he was saying his departmental monies and</p> <p>24 then he was asked, okay, where does that come</p>

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<p>1 from, and he couldn't answer that and said, well,</p> <p>2 I don't know. And the problem is --</p> <p>3 MS. THOMPSON:</p> <p>4 Q That's -- that's just not right.</p> <p>5 A Okay. Can we look at it?</p> <p>6 Q And I don't have his deposition here.</p> <p>7 But to put as your heading "Dr. Saed's</p> <p>8 Plaintiff-Funded Research" without really knowing</p> <p>9 the situation is -- doesn't sound like something</p> <p>10 you would write in a paper.</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A No.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Does it?</p> <p>16 A In a peer-review paper?</p> <p>17 Q Right.</p> <p>18 A No. But this is not a peer-review</p> <p>19 paper.</p> <p>20 Q Well, did you not --</p> <p>21 A The fact that he has plaintiff-funded</p> <p>22 research and hasn't really revealed it is a huge</p> <p>23 issue.</p> <p>24 Q What -- what's your basis for saying he</p>	<p>1 A Yeah.</p> <p>2 Q -- the published manuscript.</p> <p>3 (DEPOSITION EXHIBIT NUMBER 28</p> <p>4 WAS MARKED FOR IDENTIFICATION.)</p> <p>5 MS. THOMPSON:</p> <p>6 Q Have you seen that?</p> <p>7 A I have seen this, yes.</p> <p>8 Q And you're talking about the conflict</p> <p>9 of interest statement; correct?</p> <p>10 A Yes.</p> <p>11 Q Doctor -- I'm sorry. Exhibit 28 is his</p> <p>12 manuscript.</p> <p>13 And the declaration of conflicting</p> <p>14 interests.</p> <p>15 A Uh-huh.</p> <p>16 Q "Dr. Saed has served as a paid</p> <p>17 consultant and expert witness in the talcum</p> <p>18 litigation."</p> <p>19 Is -- is that a reason to make the</p> <p>20 heading of your report "Dr. Saed's</p> <p>21 Plaintiff-Funded Research"?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A Well, I think -- so I guess the</p>
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<p>1 hasn't revealed it?</p> <p>2 A It's not on the manuscript.</p> <p>3 Q The manuscript that's published?</p> <p>4 A Yeah.</p> <p>5 Q Well, let's look at the manuscript.</p> <p>6 So is your criticism that it's not on</p> <p>7 the manuscript or that it's plaintiff-funded</p> <p>8 research?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A Well, it's two. Yeah.</p> <p>12 MS. THOMPSON:</p> <p>13 Q Because there's nothing in that heading</p> <p>14 that says this research -- I just -- I just don't</p> <p>15 understand the heading "Dr. Saed's</p> <p>16 Plaintiff-Funded Research."</p> <p>17 A So I think there's two components</p> <p>18 there. One is I think it is an issue that --</p> <p>19 that there's dollars flowing to do some of that</p> <p>20 research. I think that raises an issue of how</p> <p>21 objective he is.</p> <p>22 And then a second issue is at a minimum</p> <p>23 it should be revealed.</p> <p>24 Q Now, this is --</p>	<p>1 question is: Is this accurate? This was not on</p> <p>2 the preprint. This was not on the --</p> <p>3 MS. THOMPSON:</p> <p>4 Q This is what's published; right?</p> <p>5 A That's not a preprint.</p> <p>6 Q Do you know what correspondence</p> <p>7 Dr. Saed -- or what -- what are you speaking of?</p> <p>8 The submission to --</p> <p>9 A The paper was submitted to GYN ONC and</p> <p>10 rejected, and then the paper was submitted to --</p> <p>11 this is Reproductive Sciences. And those --</p> <p>12 again, do we have a copy of that? I got the</p> <p>13 preprint which stated -- which said none of that.</p> <p>14 Q Okay. We'll get to that in a minute.</p> <p>15 A This was only put on afterwards.</p> <p>16 Q Do you have any -- do you have any</p> <p>17 knowledge of the conversations that Dr. Saed had</p> <p>18 with the editors of either journal as to what</p> <p>19 should go on his conflict of interest statement</p> <p>20 with the situation that he was in?</p> <p>21 Do you have any knowledge of that</p> <p>22 whatsoever?</p> <p>23 A Verbal conversations.</p> <p>24 Q Written and verbal conversations.</p>

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<p style="text-align: right;">Page 322</p> <p>1 A So verbal conversations, I don't know. 2 I'm not there. The written interactions between 3 the journals, we had copies of. 4 Q And you think what you saw was 5 sufficient enough for you to state "Dr. Saed's 6 Plaintiff-Funded Research" in this report? 7 A I think so, yeah. It's a big issue. 8 Q Wouldn't a scientist want to look at 9 the research before they call it plaintiff-funded 10 research? 11 MS. CURRY: 12 Object to the form. 13 MS. THOMPSON: 14 Q Doesn't that automatically indicate 15 that you think the research is biased? 16 A Well, again, I -- so as this document 17 evolved, I looked at the science and I -- I was 18 chagrined. That then put this into context. I 19 think -- I think it's a concern. 20 Q Well, couldn't you have just said 21 "Dr. Saed's Research" and then written your 22 comments without making the heading 23 "Plaintiff-Funded Research"? 24 MS. CURRY:</p>	<p style="text-align: right;">Page 324</p> <p>1 actual research in the lab, is that -- 2 A I can't quite -- 3 MS. CURRY: 4 Object to the form. 5 A I can't quite remember. 6 MS. THOMPSON: 7 Q Okay. 8 A But -- 9 Q So -- 10 A It was a big position. 11 Q So do you think that heading is fair? 12 A I think it is. 13 Q Do you remember Dr. Saed's testimony 14 that he would have been -- that he would have 15 been happy to do the same research had 16 Johnson & Johnson approached him on the same 17 topic? 18 MS. CURRY: 19 Object to the form. 20 A I can't remember. Do you have the 21 deposition? 22 MS. THOMPSON: 23 Q I don't. 24 A Okay.</p>
<p style="text-align: right;">Page 323</p> <p>1 Object to the form. 2 A I could have. 3 MS. THOMPSON: 4 Q Isn't there plenty of research being 5 done that's funded by various entities that's 6 quality research? 7 A So there's a broad spectrum of -- 8 Q Answer my question. Isn't there a lot 9 of research that's being done funded by various 10 entities that's quality research? 11 A As a general statement? 12 Q Uh-huh. 13 A Yes. 14 Q Yes. 15 And funding has to come from somewhere; 16 correct? 17 MS. CURRY: 18 Object to the form. 19 A Can't work without money. 20 MS. THOMPSON: 21 Q And, again, you may not remember this 22 from Dr. Saed's deposition, but his testimony 23 that there was no money coming from the 24 litigation into his lab funds which paid for the</p>	<p style="text-align: right;">Page 325</p> <p>1 Q You don't remember that he said his 2 research would have been the same and he would 3 have been willing to do it for Johnson & Johnson? 4 MS. CURRY: 5 Object to the form. 6 A I can't remember it. 7 MS. THOMPSON: 8 Q To your knowledge, has 9 Johnson & Johnson approached any researcher about 10 doing studies that would help understand whether 11 talcum powder has any molecular effects? 12 MS. CURRY: 13 Object to the form. 14 A He certainly didn't approach me. But 15 I -- I think I recall in the past they've had a 16 J & J-funded study, I think, which was 17 acknowledged on the paper. 18 MS. THOMPSON: 19 Q A molecular study? 20 A I can't say that. 21 Q If you had that, I would certainly like 22 to see it. So, to your knowledge, 23 Johnson & Johnson hasn't asked -- has not asked 24 any researchers to look at the molecular effects</p>

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<p>1 of talcum powder in cell culture?</p> <p>2 A Outside the company, right?</p> <p>3 Q How about inside the company?</p> <p>4 A I don't know. I don't know what goes</p> <p>5 on there.</p> <p>6 Q Did you ask the attorneys --</p> <p>7 A No.</p> <p>8 Q -- if Johnson & Johnson had done any</p> <p>9 studies that you could look at and --</p> <p>10 A No.</p> <p>11 Q -- criticize in the same way you did</p> <p>12 Dr. Saed?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A Well, I wouldn't rely on those, the</p> <p>16 internal documents. I would have to know the</p> <p>17 context.</p> <p>18 MS. THOMPSON:</p> <p>19 Q Well, can't you --</p> <p>20 A But this is -- this is peer-reviewed.</p> <p>21 Q Can't you find the context of -- of</p> <p>22 what studies have been done by the company?</p> <p>23 A I think that would be hard.</p> <p>24 Q So it would be of no interest to you</p>	<p>1 A No.</p> <p>2 Q Did you have any conversations by</p> <p>3 email, text or phone with the editors or any</p> <p>4 other representatives of the journal regarding</p> <p>5 this paper?</p> <p>6 A No.</p> <p>7 Q Did you have any conversations with</p> <p>8 Johnson & Johnson regarding the manuscript while</p> <p>9 it was under review?</p> <p>10 A No.</p> <p>11 Q Did you have any conversations with any</p> <p>12 of the reviewers on the paper?</p> <p>13 A I don't know who the reviewers were.</p> <p>14 Q Okay.</p> <p>15 A Yeah.</p> <p>16 Q But you have seen the reviewer comments</p> <p>17 from GYN Oncology; correct?</p> <p>18 A I did.</p> <p>19 Do we have a copy?</p> <p>20 MS. CURRY:</p> <p>21 I think she's --</p> <p>22 MS. THOMPSON:</p> <p>23 Yeah, I'm --</p> <p>24 (DEPOSITION EXHIBIT NUMBER 29 WAS</p>
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<p>1 one way or the other whether Johnson & Johnson</p> <p>2 had done any molecular studies on talcum powder</p> <p>3 and its effect on -- on tissue or cells?</p> <p>4 A Correct.</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A Correct.</p> <p>8 MS. THOMPSON:</p> <p>9 Q When did you -- is the paper that we</p> <p>10 just marked as exhibit --</p> <p>11 A 28.</p> <p>12 Q -- 28, was that paper peer-reviewed?</p> <p>13 A This is a peer-review journal.</p> <p>14 Q And when did you first see the</p> <p>15 unpublished manuscript?</p> <p>16 A I am gonna really -- I'm stretching on</p> <p>17 this. I think it was about -- let's say a month</p> <p>18 or two before this.</p> <p>19 Q Okay. So a couple months ago?</p> <p>20 A Yeah.</p> <p>21 Q Do you review papers for Gynecologic</p> <p>22 Oncology?</p> <p>23 A I do.</p> <p>24 Q Were you asked to review this paper?</p>	<p>1 MARKED FOR IDENTIFICATION.)</p> <p>2 MS. THOMPSON:</p> <p>3 Q I'm gonna go ahead and mark Exhibit 29.</p> <p>4 29 will be the reviewer comments from the journal</p> <p>5 Gynecologic Oncology.</p> <p>6 A Uh-huh.</p> <p>7 Q And again, that journal is the</p> <p>8 journal -- or maybe we haven't discussed this --</p> <p>9 it's the journal for SGO, the Society of</p> <p>10 Gynecologic Oncologists; correct?</p> <p>11 A Correct.</p> <p>12 Q Did I give you a highlighted copy?</p> <p>13 A You did, actually. It's very helpful.</p> <p>14 Q Let me switch that. I'm sure it was.</p> <p>15 Actually, it probably wasn't.</p> <p>16 A I've seen these before.</p> <p>17 (DEPOSITION EXHIBIT NUMBER 30 WAS</p> <p>18 MARKED FOR IDENTIFICATION.)</p> <p>19 MS. THOMPSON:</p> <p>20 Q And then I'm gonna also, at the same</p> <p>21 time, give you Exhibit 30, which is the reviewer</p> <p>22 comments from Reproductive Sciences.</p> <p>23 A All right.</p> <p>24 Q Both are peer-reviewed journals, as you</p>

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<p>1 mentioned; right?</p> <p>2 A Yes. Difference in impact, but both</p> <p>3 peer review.</p> <p>4 Q And they have a -- a different audience</p> <p>5 readership, too, wouldn't you agree?</p> <p>6 A I would agree, yes.</p> <p>7 MS. CURRY:</p> <p>8 Do you have another copy of Exhibit 30?</p> <p>9 MS. THOMPSON:</p> <p>10 Yes. I'm sorry.</p> <p>11 MS. CURRY:</p> <p>12 Thank you.</p> <p>13 MS. THOMPSON:</p> <p>14 That good?</p> <p>15 MS. CURRY:</p> <p>16 Yes.</p> <p>17 MS. THOMPSON:</p> <p>18 Q In your report, you make the statement</p> <p>19 "Unsurprisingly, this manuscript has serious</p> <p>20 methodologic, experimental and analysis flaws."</p> <p>21 A I'm sorry. Are you in the beginning of</p> <p>22 this last paragraph of 19?</p> <p>23 Q No.</p> <p>24 A No?</p>	<p>1 Q Reading the letter to Dr. Saed:</p> <p>2 "Your paper, referenced above, has now</p> <p>3 been reviewed by at least two reviewers -- has</p> <p>4 now been reviewed by at least two experts in the</p> <p>5 field and the editors. Based on the reviewer</p> <p>6 comments, we must inform you that while your work</p> <p>7 is not without merit, we are unable to accept</p> <p>8 your manuscript for publication in Gynecologic</p> <p>9 Oncology. In the last year we have seen a</p> <p>10 significant increase in the number of manuscripts</p> <p>11 submitted to the journal, and, as a result, we</p> <p>12 are now accepting less than 20 percent of the</p> <p>13 manuscripts submitted to the Gynecologic</p> <p>14 Oncology."</p> <p>15 Certainly in that first paragraph there</p> <p>16 were -- there was no language that resembles this</p> <p>17 manuscript has serious methodologic, experimental</p> <p>18 and analysis flaws, is there?</p> <p>19 A No.</p> <p>20 Q The second paragraph, "We have attached</p> <p>21 the comments of the reviewers below in order for</p> <p>22 you to understand the basis for our decision. We</p> <p>23 hope that their thoughtful comments will help you</p> <p>24 in your future studies and possibly with</p>
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<p>1 Q It's in another spot. Let me find it.</p> <p>2 A Maybe it's under the paper.</p> <p>3 Q Yeah. Page 24.</p> <p>4 A Yep. Yeah.</p> <p>5 Q "Unsurprisingly, this manuscript has</p> <p>6 serious methodologic, experimental and analysis</p> <p>7 flaws."</p> <p>8 A Uh-huh.</p> <p>9 Q Did you see any language to that effect</p> <p>10 in the peer-reviewers' comments?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A One second.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Well, let me just ask you.</p> <p>16 Did those words appear in the reviewer</p> <p>17 comments?</p> <p>18 A No, I don't think so.</p> <p>19 Q Okay.</p> <p>20 A Yeah.</p> <p>21 Q So let's -- I want to actually go</p> <p>22 through the reviewer comments. We'll start with</p> <p>23 Gynecologic Oncology.</p> <p>24 A Yep.</p>	<p>1 submission to another journal.</p> <p>2 "Please note that a revised version of</p> <p>3 the current manuscript should not be submitted</p> <p>4 for another review to Gynecologic Oncology."</p> <p>5 There's certainly no language in that</p> <p>6 paragraph that resembles serious methodologic,</p> <p>7 experimental and analysis flaws, is there?</p> <p>8 A No.</p> <p>9 Q And the reviewers actually encouraged</p> <p>10 Dr. Saed to submit the article to another</p> <p>11 journal; correct?</p> <p>12 A Well, this isn't the reviewer. This is</p> <p>13 the editor.</p> <p>14 Q The editor?</p> <p>15 A Yeah.</p> <p>16 Q The editors?</p> <p>17 A Yeah. And this is boilerplate. You'd</p> <p>18 always get this. They're not --</p> <p>19 Q Well, I'm just asking you for the --</p> <p>20 for what the -- what the letter says.</p> <p>21 A Yeah. Yeah.</p> <p>22 Q "The critique of this letter in no way</p> <p>23 implies a lack of interest in this area of</p> <p>24 research and we invite you to submit your future</p>

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<p style="text-align: right;">Page 334</p> <p>1 work to the journal."</p> <p>2 Is that what the letter from</p> <p>3 Dr. Bristow, the editor says?</p> <p>4 A Correct.</p> <p>5 Q And, in fact, Dr. Saed has published</p> <p>6 several times in this journal previously.</p> <p>7 Are you aware of that?</p> <p>8 A Yeah. I believe so, yeah.</p> <p>9 Q So let's go ahead and go through the --</p> <p>10 the reviewer comments. Reviewer number 1 --</p> <p>11 And, as you testified, you don't know</p> <p>12 who these reviewers are; correct?</p> <p>13 A I don't.</p> <p>14 Q Reviewer 1, in his summary of</p> <p>15 Dr. Saed's paper, says "The stated objective of</p> <p>16 the study by Fletcher and colleagues is to</p> <p>17 determine the effects of talc on expression of</p> <p>18 key inflammatory and redox markers in ovarian</p> <p>19 cancer and normal cell lines. Normal ovarian and</p> <p>20 EOC cells were treated with various doses of talc</p> <p>21 for 48 hours. Levels of CA-125 and selected key</p> <p>22 redox enzymes were measured using realtime P --</p> <p>23 RT-PCR and ELISA."</p> <p>24 Is that an accurate statement of what</p>	<p style="text-align: right;">Page 336</p> <p>1 MS. THOMPSON:</p> <p>2 Q Right.</p> <p>3 A Yeah.</p> <p>4 Q "This is an important but controversial</p> <p>5 topic in need of rigorous scientific inquiry."</p> <p>6 Why is this a controversial topic, in</p> <p>7 your mind?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 MS. THOMPSON:</p> <p>11 Q Or is it a controversial topic to you?</p> <p>12 A I would assume they're referring to the</p> <p>13 potential role of talc in ovarian cancer. But</p> <p>14 I'm -- again, it's speculative.</p> <p>15 Q Okay.</p> <p>16 A I'm guessing.</p> <p>17 Q So you wouldn't know why it would be</p> <p>18 considered controversial?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A No. Not -- not in -- no, vis-à-vis</p> <p>22 from what the reviewer's saying.</p> <p>23 MS. THOMPSON:</p> <p>24 Q "The current in vitro study does" --</p>
<p style="text-align: right;">Page 335</p> <p>1 the objective of the study was?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 A I think that's -- I think that's a</p> <p>5 little terse, but it covers the bases.</p> <p>6 MS. THOMPSON:</p> <p>7 Q And then beginning with the reviewer</p> <p>8 comments, reviewer number 1 says "Overall, this</p> <p>9 is a well-written manuscript and the conclusions</p> <p>10 are supported by the results."</p> <p>11 Do you disagree with that comment by</p> <p>12 reviewer number 1?</p> <p>13 A That's very generous. I don't agree</p> <p>14 with it. Particularly the latter part.</p> <p>15 Q But at least that's what the</p> <p>16 reviewer --</p> <p>17 A Correct.</p> <p>18 Q -- who was -- you would think was</p> <p>19 chosen because of their expertise in the field,</p> <p>20 those are the reviewer comments regarding</p> <p>21 Dr. Saed's paper; correct?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A For reviewer 1.</p>	<p style="text-align: right;">Page 337</p> <p>1 reading on, "The current in vitro study does</p> <p>2 provide novel information, but there are also</p> <p>3 some important limitations described below."</p> <p>4 Would you agree that it's common to</p> <p>5 have a back-and-forth with a reviewer and author</p> <p>6 before publication of a paper?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Some papers are accepted de novo, but</p> <p>10 it's unusual. Usually there are criticisms and,</p> <p>11 then you'd have to revise. Sometimes if it's</p> <p>12 Cancer Cell, it goes back and forth for two</p> <p>13 years.</p> <p>14 MS. THOMPSON:</p> <p>15 Q The reviewer number 1 in -- in the</p> <p>16 bullet point number 1, said "The significance of</p> <p>17 the study would be greatly enhanced if a mouse</p> <p>18 model corroborated the cell line findings."</p> <p>19 I would -- I'm guessing you're gonna</p> <p>20 agree with that statement?</p> <p>21 A I do.</p> <p>22 Q But you would also agree, I think, that</p> <p>23 oftentimes you -- a researcher would start with</p> <p>24 an in vitro study; correct?</p>

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<p>1 A Frequently.</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 MS. THOMPSON:</p> <p>5 Q And what would the reasons for that be?</p> <p>6 A It's usually easier.</p> <p>7 Q Less costly?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A By definition.</p> <p>11 MS. THOMPSON:</p> <p>12 Q And could be completed in less time?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A Usually, yeah.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Do you -- do you have any idea or</p> <p>18 knowledge of what experiments Dr. Saed is</p> <p>19 currently doing in the -- in the area of talcum</p> <p>20 powder and its biologic effects?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A I don't.</p> <p>24 MS. THOMPSON:</p>	<p>1 A I'm not done with my response.</p> <p>2 So let me finish the first statement.</p> <p>3 Q Okay.</p> <p>4 A I think if you could show a phenom- --</p> <p>5 if you could show the biologic effects in a mouse</p> <p>6 model, then it's much stronger data, regardless</p> <p>7 of the cell lines.</p> <p>8 I don't -- I would agree I don't think</p> <p>9 Dr. Saed said much about CA-125 being -- being</p> <p>10 involved in ovarian cancer development, and</p> <p>11 that's the point. I don't understand, and I</p> <p>12 think a lot of other of us who have looked at</p> <p>13 this, don't understand what the value is of the</p> <p>14 increase in CA-125.</p> <p>15 Q Do you know that when Dr. Saed</p> <p>16 presented the initial data at the meeting, that</p> <p>17 the attendees requested that he perform CA-125</p> <p>18 and that's why he performed it? Do you remember</p> <p>19 seeing that in his deposition?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A I didn't see that. Which meeting was</p> <p>23 this? Do you know?</p> <p>24 MS. THOMPSON:</p>
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<p>1 Q In this reviewer's opinion, "The cell</p> <p>2 line studies alone and the increase in CA-125,</p> <p>3 while intriguing, are not sufficiently</p> <p>4 convincing."</p> <p>5 Would you agree with that statement?</p> <p>6 A Absolutely.</p> <p>7 Q And so a mouse model corroboration of</p> <p>8 the findings would be -- would enhance the</p> <p>9 results; correct?</p> <p>10 A Not from my perspective. And I'm not</p> <p>11 so sure this reviewer's implying that. I think</p> <p>12 there's a real question anything can be</p> <p>13 interpreted from the cell line studies, and any</p> <p>14 increase in CA-125 is meaningless because CA-125</p> <p>15 is a marker.</p> <p>16 So I think --</p> <p>17 Q Well, wait a minute.</p> <p>18 Did Dr. Saed say anything about</p> <p>19 CA-125 --</p> <p>20 MS. CURRY:</p> <p>21 Are you done with your response?</p> <p>22 MS. THOMPSON:</p> <p>23 Q -- being the significance with the</p> <p>24 findings?</p>	<p>1 Q SRI, 2018.</p> <p>2 A Okay.</p> <p>3 Q Society of Reproductive Investigators.</p> <p>4 A And did they indicate -- anybody</p> <p>5 indicate what the purpose of that was?</p> <p>6 Q I can't tell you that.</p> <p>7 But, listen, I'm -- I'm just reading</p> <p>8 the reviewer's comments --</p> <p>9 A Yeah.</p> <p>10 Q -- without either one of us trying to</p> <p>11 speculate on what he means.</p> <p>12 But the statement is "The significance</p> <p>13 of this study would be greatly enhanced if a</p> <p>14 mouse model corroborated the cell line findings."</p> <p>15 So there were cell line findings to be</p> <p>16 corroborated; correct?</p> <p>17 A Correct.</p> <p>18 Q The reviewer number 1 also said "The</p> <p>19 significance of SNP alterations" -- that's SNP,</p> <p>20 all capitalized -- "should be further clarified."</p> <p>21 And I think you would agree with that;</p> <p>22 correct?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>

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<p>1 A I strongly agree with that.</p> <p>2 MS. THOMPSON:</p> <p>3 Q And the viewer -- reviewer commented,</p> <p>4 "The first bulleted highlight, Oxidative Stress,</p> <p>5 is a key mechanism to the initiation and</p> <p>6 progression of ovarian cancer is not supported by</p> <p>7 this investigation and should be omitted."</p> <p>8 Does the reviewer comment on why that</p> <p>9 should be -- that line should be omitted, other</p> <p>10 than it wasn't supported by this investigation</p> <p>11 with talcum powder?</p> <p>12 A No. It would be speculative. It's --</p> <p>13 it's as you read it.</p> <p>14 Q Okay. Do you know that -- that</p> <p>15 virtually that exact statement has been published</p> <p>16 in this same journal in the past by Dr. Saed and</p> <p>17 others?</p> <p>18 MS. CURRY:</p> <p>19 Object to the form.</p> <p>20 A As a stand-alone statement?</p> <p>21 MS. THOMPSON:</p> <p>22 Q Yeah. Yes.</p> <p>23 A Yeah. I don't think that addresses</p> <p>24 what the reviewer is saying.</p>	<p>1 Object to the form.</p> <p>2 A And it's -- and it's -- I don't know --</p> <p>3 just one comment that it's more detailed, which</p> <p>4 makes someone like me as a third party look at</p> <p>5 and say, well, they actually read the paper. I'd</p> <p>6 worry a little about if reviewer 1 didn't read it</p> <p>7 carefully enough.</p> <p>8 MS. THOMPSON:</p> <p>9 Q But you have no idea what he did?</p> <p>10 A I've been speculating all day.</p> <p>11 Q Okay. All right. And then the first</p> <p>12 sentence of reviewer number 2, "While the authors</p> <p>13 compellingly show changes in several key enzymes</p> <p>14 recognizing redox potential in cells exposed to</p> <p>15 talc, their data do not show, despite the</p> <p>16 author's claim, any evidence that these cells are</p> <p>17 transformed."</p> <p>18 Do you agree with reviewer number 2 in</p> <p>19 that statement?</p> <p>20 A I agree.</p> <p>21 Q Second sentence, "Specifically, no</p> <p>22 experiments documenting changes in cell survival</p> <p>23 proliferation are resistant to apoptosis have</p> <p>24 been performed."</p>
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<p>1 Q Yeah.</p> <p>2 A The reviewer's saying it's not</p> <p>3 supported by --</p> <p>4 Q And that's the point I was trying to</p> <p>5 make.</p> <p>6 So -- so you would agree that it</p> <p>7 doesn't sound like it's the statement that's at</p> <p>8 issue; it's whether the talcum powder studies are</p> <p>9 supportive of that statement?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A Well, the way it's phrased here -- the</p> <p>13 way it's phrased here, I agree. Yeah.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Let's go to reviewer number 2.</p> <p>16 A Uh-huh.</p> <p>17 Q And reviewer number 2 gives a similar</p> <p>18 summary, perhaps with a little more detail.</p> <p>19 A Yeah.</p> <p>20 Q But would you agree it's an accurate</p> <p>21 description of what the objectives of the study</p> <p>22 were?</p> <p>23 A It is.</p> <p>24 MS. CURRY:</p>	<p>1 And that's correct; right?</p> <p>2 A So he does show what he thinks is</p> <p>3 proliferation, if I recall correctly. I believe</p> <p>4 it's an MMT -- MTT assay.</p> <p>5 Q Well, those experiments were done</p> <p>6 following reviewer number 2's recommendation. Is</p> <p>7 that your understanding?</p> <p>8 A Well, I --</p> <p>9 Q In the --</p> <p>10 A Yeah.</p> <p>11 Q In the first manuscript. Do you</p> <p>12 remember that?</p> <p>13 A You could be right. I don't have it</p> <p>14 pre- -- I don't have that version in front of me.</p> <p>15 Q You may have to just take my word for</p> <p>16 that.</p> <p>17 MS. CURRY:</p> <p>18 I have a copy of it if you need it.</p> <p>19 MS. THOMPSON:</p> <p>20 No. It's not too -- I don't think it's</p> <p>21 too much --</p> <p>22 A But I can say, in particular, cell</p> <p>23 survival resistant apoptosis, I don't think has</p> <p>24 been effectively performed.</p>

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<p>1 MS. THOMPSON: 2 Object. That didn't answer a question. 3 Nonresponsive. 4 Q Next sentence, "Consequently, neither 5 tumor initiation nor progression is documented in 6 this study as opposed to the statement in 7 highlight number 1 and elsewhere." 8 "While changes in redox potential play 9 an important role in tumor biology in general, 10 the present data are insufficient to back up the 11 claim that talc is central to the development of 12 ovarian cancer." 13 Did Dr. Saed make a claim that talcum 14 is central to the development of ovarian cancer, 15 that you recall? 16 A I don't recall him saying that. 17 Q I don't either. 18 "Other comments: The introduction 19 should be better organized with shorter 20 description of the general features of ovarian 21 cancer, replaced by a brief overview of redox 22 proteins in cancer, followed by a discussion of 23 their role in ovarian cancer." 24 That's more a style issue. Would you</p>	<p>1 Q Where in -- where in Dr. Saed's paper 2 does it say this paper shows talcum powder 3 transforms ovarian cells? 4 A Do we have the original? 5 Q We're looking at the published 6 manuscript. 7 MS. CURRY: 8 But the comments are based on the -- 9 A This is the one published in -- and you 10 already told me he changed some of the 11 experiments. 12 MS. THOMPSON: 13 Q Was -- shouldn't your critique be the 14 published paper? 15 A Well, you're asking me to review this; 16 right? 17 Q Okay. We can pull out the -- we can 18 pull out the published manuscript. 19 But certainly in the published paper, 20 there are no claims that cells are transformed, 21 are there? 22 A Well, let's take a look. 23 Q It's certainly not in the abstract or 24 in the conclusion -- in the summary, is it?</p>
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<p>1 agree? 2 MS. CURRY: 3 Object to the form. 4 A Make it -- make it more readable, yeah. 5 MS. THOMPSON: 6 Q And, then, the -- finally, "The fact 7 that SNPs were changed following such short 8 exposure to talcum is surprising and makes one 9 wonder what the biological effects of such change 10 might be." 11 And those are the reviewer comments 12 from Gynecologic Oncology; correct? 13 A Correct. 14 Q Did the peer-reviewers raise concerns 15 about Dr. Saed's, in your words, unsubstantiated 16 assumptions? 17 A Well, I -- I think it's implicit in 18 some of the comments. 19 Q That there are unsubstantiated 20 assumptions? 21 A So -- so I think if you read the second 22 paragraph of the second reviewer -- remember, 23 this paper basically says that talc transforms 24 ovarian cancer cells.</p>	<p>1 A I'm just getting through the discussion 2 a little bit. It may be -- may be buried in 3 there or may be an implication that the soft 4 argarose cloning is reflective of only the 5 changes. 6 Q Dr. Saed's paper does not claim that 7 the cells were transformed, does it? 8 A Let me look through it, then. 9 Q Okay. Let's go off the record. 10 VIDEOGRAPHER: 11 Off the record at 4:23 p.m. 12 (OFF THE RECORD.) 13 VIDEOGRAPHER: 14 We're back on the record at 4:24 p.m. 15 A Page 7 on the bottom. "In this study 16 we've shown that talc enhances cellular 17 proliferation, induces inhibition of apoptosis 18 and C-cells" -- 19 MS. CURRY: 20 Gotta go slow for Lois. 21 THE WITNESS: 22 Oh. 23 -- "but, more importantly, in normal 24 cells, suggesting talc is a stimulus to the</p>

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<p style="text-align: right;">Page 350</p> <p>1 development of an oncogenic phenotype." 2 MS. THOMPSON: 3 Q That doesn't say the cells were 4 transformed, does it? 5 A I think for those of us in the field 6 that implies transformation. 7 Q Well, it certainly doesn't state -- 8 state cells were transformed, as you stated 9 earlier. 10 MS. CURRY: 11 Object to the form. 12 MS. THOMPSON: 13 Q Did the reviewers have -- raise any 14 concerns about serious flaws in methodology? 15 A You know, the significance of SNP 16 alteration should be further clarified. That's a 17 pleasant way of saying I don't understand what 18 you're doing. 19 Q I'm asking did the peer-reviewers raise 20 concerns about serious flaws in methodology? 21 MS. CURRY: 22 Object to the form. 23 A In those terms? 24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 352</p> <p>1 MS. CURRY: 2 Object to the form. 3 A Correct. 4 MS. THOMPSON: 5 Q And wouldn't that be the flaws in the 6 analysis that you're referring to? 7 A I don't know what that refers to in 8 vis-à-vis my statement. 9 Q Did the reviewers state that any of the 10 cell line findings appeared to be inaccurate? 11 A No. 12 Q Did the reviewers state that the wrong 13 cell lines were used? 14 A No. 15 Q Did the reviewers state that the doses 16 were inappropriate? 17 A No. 18 Q Did the reviewers state that the CA-125 19 findings were irrelevant? 20 MS. CURRY: 21 Object to the form. 22 A Increase in CA-125 while intriguing are 23 not sufficiently convincing to make it relevant 24 or not.</p>
<p style="text-align: right;">Page 351</p> <p>1 Q Yes, in those terms. 2 A No. 3 Q Did the peer-reviewers raise concerns 4 about serious flaws in the experiments? 5 A In those terms? 6 Q Right. 7 A No. 8 Q Did the peer-reviewers raise serious 9 concerns about flaws in the analysis? 10 A No. 11 Q And, in fact, peer-reviewer number 1 12 explicitly stated that "The conclusions are 13 supported by the results." 14 Right? 15 MS. CURRY: 16 Object to the form. 17 A They rejected the paper. 18 MS. THOMPSON: 19 Q I -- that wasn't my question. 20 The question was -- I mean, my question 21 was that the reviewer number 1 specifically 22 states "The conclusions are supported by the 23 results." 24 Correct?</p>	<p style="text-align: right;">Page 353</p> <p>1 MS. THOMPSON: 2 Q But the reviewer certainly didn't say 3 they're irrelevant? 4 A Didn't use those terms. 5 Q And intriguing would at least mean that 6 the reviewer 1 thought they were of some 7 interest. Wouldn't you agree? 8 MS. CURRY: 9 Object to the form. 10 A Some interest. Some interest. 11 MS. THOMPSON: 12 Q The reviewer did ask for clarification 13 of the significance of SNPs. Did the reviewer 14 state that the SNP findings were irrelevant? 15 A Not in those terms. 16 Q Did the reviewer state that the 17 methodology used to test for the SNPs was flawed? 18 A You know, again, they're seeking 19 clarification. That suggests to me that they 20 have a problem with the way it was done. 21 Wouldn't they -- 22 Q Did -- did the reviewer state the 23 methodology used to test the SNPs was flawed? 24 MS. CURRY:</p>

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<p style="text-align: right;">Page 354</p> <p>1 Sorry. You keep cutting off his answer</p> <p>2 when he's not finished.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Were you finished?</p> <p>5 A Well, I'm just asking what are they</p> <p>6 trying to clarify?</p> <p>7 Q I'm just asking you did -- was there a</p> <p>8 comment that the methodology for testing the SNPs</p> <p>9 was flawed?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A They do not say that.</p> <p>13 MS. THOMPSON:</p> <p>14 Q Okay. Did the reviewers state that the</p> <p>15 SNP data was in a accurate?</p> <p>16 A I don't think they know. It has to be</p> <p>17 clarified.</p> <p>18 Q And are you aware that the same SNP</p> <p>19 data was submitted to SGO as an abstract and</p> <p>20 recently presented at the annual meeting?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A The one --</p> <p>24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 356</p> <p>1 Q Did the reviewer --</p> <p>2 A I hope not.</p> <p>3 Q Did either reviewer state that the data</p> <p>4 was poor?</p> <p>5 MS. CURRY:</p> <p>6 Object to the form.</p> <p>7 A Not in that specific term.</p> <p>8 MS. THOMPSON:</p> <p>9 Q Let's look at the reviewer from</p> <p>10 Reproductive Sciences.</p> <p>11 Are you going to give me yours?</p> <p>12 A I've got this pretty much memorized.</p> <p>13 MS. EVERETT:</p> <p>14 Did we put it back in the folder? Here</p> <p>15 is one.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Okay. And the paper was accepted at</p> <p>18 Reproductive Sciences. Is that your</p> <p>19 understanding, since it was eventually published?</p> <p>20 A Yes.</p> <p>21 Q Did the reviewers at Reproductive</p> <p>22 Sciences make any statements regarding flawed</p> <p>23 methodology, experiments, or analysis?</p> <p>24 MS. CURRY:</p>
<p style="text-align: right;">Page 355</p> <p>1 Q As opposed to a presentation?</p> <p>2 A The one in Honolulu -- the one in</p> <p>3 Honolulu --</p> <p>4 Q Yes.</p> <p>5 A -- Hawaii? Yeah. Yes.</p> <p>6 Q Did you see that poster?</p> <p>7 A No.</p> <p>8 Q Did you speak with the -- the authors</p> <p>9 of the abstract and the paper?</p> <p>10 A No.</p> <p>11 Q Would that have been of interest to you</p> <p>12 to -- to speak with the researchers?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A Yeah. So the poster section conflicted</p> <p>16 with everything else I could do. I didn't see</p> <p>17 any posters. But I think given my role on this,</p> <p>18 I probably would not have gone, under any</p> <p>19 circumstances.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Do you have any knowledge as to whether</p> <p>22 either of these reviewers is a Johnson & Johnson</p> <p>23 consultant or expert?</p> <p>24 A I have no -- no idea.</p>	<p style="text-align: right;">Page 357</p> <p>1 Object to the form.</p> <p>2 A I'm sorry. I only see one reviewer;</p> <p>3 right?</p> <p>4 MS. THOMPSON:</p> <p>5 Q We only have comments from one</p> <p>6 reviewer. That's correct.</p> <p>7 A Yeah. And -- and they don't make that</p> <p>8 comment.</p> <p>9 Q So I want to just go through Dr. Saed's</p> <p>10 published paper --</p> <p>11 A Uh-huh.</p> <p>12 Q -- and discuss what was done in this --</p> <p>13 just from the materials and methods. We're not</p> <p>14 in results yet. Okay?</p> <p>15 So Dr. Saed used the following cell</p> <p>16 lines: SKOV3, A2780, TOV11 -- or 112D. And</p> <p>17 those are all ovarian cancer cell lines; correct?</p> <p>18 A There is significant question about the</p> <p>19 origin of 2780.</p> <p>20 Q Okay.</p> <p>21 A It may --</p> <p>22 Q But it is a cancerous cell line?</p> <p>23 A I would accept that. Yeah.</p> <p>24 Q Okay. And, then, there are also three</p>

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<p style="text-align: right;">Page 358</p> <p>1 noncancerous cell lines. Agree? The human 2 primary normal ovarian epithelial cells from Cell 3 Biologics Chicago, the human ovarian epithelial 4 cells from Cell Biologics, and the human -- oops. 5 A Immortal one. 6 Q And the immortalized human fallopian 7 tube secretory epithelial cells, FT33, from 8 applied biologic materials. 9 Would you agree those are three 10 noncancerous cell lines? 11 A And when you're defining 12 "noncancerous," you mean they were not isolated 13 from a tumor? 14 Q Correct. 15 A Agree on that. 16 Q Again, just going through the 17 methodology, were the cells grown in media and 18 conditions following manufacturer protocol? 19 MS. CURRY: 20 Object to the form. 21 A I'm not really sure what the 22 manufacturer suggested. But I don't -- I think 23 that the way they were cultured appeared okay to 24 me.</p>	<p style="text-align: right;">Page 360</p> <p>1 MS. CURRY: 2 Object to the form. 3 A I believe so. 4 MS. THOMPSON: 5 Q And using the realtime PCR -- RT-PCR, 6 the -- the following assays were performed. Beta 7 actin for normalization of samples; right? 8 A Yes. 9 Q CAT, SOD3? 10 A Uh-huh. 11 Q GSR, GPX1, NOS2. Are those the tests 12 that were performed with PCR? 13 A Seven -- seven genes. 14 Q Yes. 15 A Including beta actin. 16 Q And -- 17 A Yes. 18 Q And by ELISA, Dr. Saed in his lab 19 tested CAT, SOD, GSR, GPX, NPO, and the CA-125 20 that we've talked about before; correct? 21 A Yes. 22 Q And Dr. Saed -- and those have all been 23 peer-reviewed and published in other studies 24 using ELISA and testing those --</p>
<p style="text-align: right;">Page 359</p> <p>1 MS. THOMPSON: 2 Q Appeared what? 3 A Okay to me. 4 Q Okay. And you'll agree that the cells 5 were seeded and treated with zero, 5, 20, or 100 6 micrograms per mil of baby powder; correct? 7 A This is in Treatment of Cells? 8 Q Yes. 9 A Correct. 10 Q And the -- so the talcum powder was 11 dissolved in DMSO; correct? 12 A I am looking for that. Do you see 13 that? 14 Q It's in Treatment of Cells also. 15 A Oh, okay. 16 Q I went out of order. 17 A Thank you. 18 Q And are you aware that these doses have 19 previously been reported in peer-reviewed 20 literature -- 21 MS. CURRY: 22 Object to -- 23 MS. THOMPSON: 24 Q -- for the study of talc?</p>	<p style="text-align: right;">Page 361</p> <p>1 MS. CURRY: 2 Object to the form. 3 A Yes. 4 MS. THOMPSON: 5 Q -- particular markers? 6 And Dr. Saed performed the TaqMan SNP 7 genotyping assay on all cell lines; correct? 8 A It's listed there. Yes. 9 Q And those were performed by the Applied 10 Genomics Technology Center At Wayne State 11 University; correct? 12 A Yes. 13 Q And is it your understanding that this 14 is a core facility? 15 MS. CURRY: 16 Object to the form. 17 A That, I don't know. But it could be. 18 MS. THOMPSON: 19 Q What is a core facility? 20 A It's generally a facility that provides 21 standard assays, and everybody shares, and they 22 charge a fee. 23 Q Is there some accreditation of core 24 facilities for quality control?</p>

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<p style="text-align: right;">Page 362</p> <p>1 A Usually it's institutional. In other 2 words, it's not an external group. But a 3 institution won't fund the core unless it's doing 4 decent work. 5 Q And Dr. Saed and his researchers then 6 performed the cell proliferation and apoptosis 7 studies using the TACS MTT self-proliferation 8 assay; correct? 9 A Yes. 10 Q And -- and cast pace 3 after treatment 11 of all the cell lines with the various doses; 12 correct? 13 A Yes. 14 Q And you'll agree that all of these 15 tests have been performed, peer-reviewed, and 16 published previously by Dr. Saed and others; 17 correct? 18 MS. CURRY: 19 Object to the form. 20 A I don't know that. But these are 21 reasonably standard. 22 MS. THOMPSON: 23 Q These are standardized -- 24 A Yeah.</p>	<p style="text-align: right;">Page 364</p> <p>1 A They're generally accepted. I -- 2 "standardized" is a difficult word because it 3 implies some sort of external review or 4 standardization. And that's not true. These are 5 kits that are -- are bought and then they're 6 implemented in the lab. You still don't know 7 whether it's really being done right, but -- 8 MS. THOMPSON: 9 Q Okay. Well it sounds like -- 10 A -- but -- but -- but they're -- we're 11 familiar with these -- 12 Q Okay. 13 A -- and there's nothing too much out of 14 the box there. 15 Q And before, you said these are 16 standardized, yeah, so I was just going back to 17 that. 18 A Right. 19 Q I think we got the answer. 20 I'm about to start a little bit 21 different area. 22 MS. THOMPSON: 23 Do we want to take a break now or do 24 you want to go for another 30 minutes or so?</p>
<p style="text-align: right;">Page 363</p> <p>1 Q -- testing methods. 2 All right. Let -- let me just ask that 3 question again because we've got a -- these are 4 standardized testing methods; correct? 5 MS. CURRY: 6 Object to the form. 7 A I don't know what you mean by 8 "standardized." These are assays that many labs 9 use. They're not being done in -- they're not 10 being done in a central CLIA-approved lab. 11 They're just being done by him and maybe a core 12 lab. 13 MS. THOMPSON: 14 Q And I was just asking the question 15 because previously it got chopped into two pieces 16 on these are standardized -- yeah, testing 17 methods, all right. So I was just trying to get 18 a single answer -- 19 A Yes. 20 Q -- was the purpose of that question. 21 So these are standardized testing 22 methods; correct? 23 MS. CURRY: 24 Object to the form.</p>	<p style="text-align: right;">Page 365</p> <p>1 MS. CURRY: 2 How much time do we have left on the 3 record? 4 VIDEOGRAPHER: 5 An hour and seven minutes. 6 MS. CURRY: 7 Do you want to take a final break now? 8 MS. THOMPSON: 9 Yeah. I'll easily finish the rest, I 10 think, in an hour and seven minutes. 11 MS. CURRY: 12 Okay. 13 MS. THOMPSON: 14 Maybe even less. 15 VIDEOGRAPHER: 16 Off the record at 4:39 p.m. 17 (OFF THE RECORD.) 18 VIDEOGRAPHER: 19 We're back on the record at 4:50 p.m. 20 MS. THOMPSON: 21 Q Dr. Birrer, I'd like to do another 22 chart with Dr. Saed's research so I can 23 understand what your opinions are regarding his 24 findings. Okay?</p>

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<p>1 A Okay.</p> <p>2 MS. CURRY:</p> <p>3 And for the record, I object to the</p> <p>4 creation of this chart.</p> <p>5 (DEPOSITION EXHIBIT NUMBER 31 WAS</p> <p>6 MARKED FOR IDENTIFICATION.)</p> <p>7 MS. CURRY:</p> <p>8 What's the exhibit number?</p> <p>9 MS. THOMPSON:</p> <p>10 And this would be Exhibit 31.</p> <p>11 Q And these are the tables taken from</p> <p>12 Dr. Saed's manuscript. Does that look right?</p> <p>13 If you want to compare, you can.</p> <p>14 A Let me just compare.</p> <p>15 MS. CURRY:</p> <p>16 This the from the published manuscript?</p> <p>17 MS. THOMPSON:</p> <p>18 Q This is from the published manuscript?</p> <p>19 A This is from Figure 1, right?</p> <p>20 Q And -- and you'll agree that these</p> <p>21 charts are generated from the raw data; correct?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A It appears so.</p>	<p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A I assume they are. I mean, in terms of</p> <p>4 they reflect the actual raw data, yeah.</p> <p>5 MS. THOMPSON:</p> <p>6 Q Right. So I'm going to put a Y --</p> <p>7 A Okay.</p> <p>8 Q -- for accurate.</p> <p>9 A Oh. You're looking at all of them?</p> <p>10 Q Oh. Do you have any --</p> <p>11 MS. CURRY:</p> <p>12 Do you have the published paper?</p> <p>13 THE WITNESS:</p> <p>14 I have it here. Right here.</p> <p>15 MS. CURRY:</p> <p>16 What exhibit is that?</p> <p>17 THE WITNESS:</p> <p>18 Yeah. Well, I'll have to say, that</p> <p>19 does look different.</p> <p>20 MS. THOMPSON:</p> <p>21 Q I can -- I'll represent that they were</p> <p>22 cut and pasted from the manuscript. So if they</p> <p>23 are different, it's a --</p> <p>24 MS. CURRY:</p>
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<p>1 MS. THOMPSON:</p> <p>2 Q And --</p> <p>3 A Although I would say --</p> <p>4 MS. GARBER:</p> <p>5 Do you have two? Because your</p> <p>6 co-counsel --</p> <p>7 MS. THOMPSON:</p> <p>8 No. That's just one copy, one exhibit.</p> <p>9 A These are -- for instance, the PCR is</p> <p>10 normalized.</p> <p>11 MS. THOMPSON:</p> <p>12 Q Okay. And this chart shows PCR and</p> <p>13 ELISA for antioxidants; right?</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 MS. THOMPSON:</p> <p>17 Q The expression of antioxidants and the</p> <p>18 activity of antioxidants CAT and SOV3; correct?</p> <p>19 A Correct.</p> <p>20 Q I want to go through this chart and</p> <p>21 have you tell me "yes" or "no" for each of these</p> <p>22 with each cell line.</p> <p>23 Do you have an opinion as to whether</p> <p>24 these results are accurate?</p>	<p>1 Okay. I'm sorry. I'm having a hard</p> <p>2 time following --</p> <p>3 A But this --</p> <p>4 MS. CURRY:</p> <p>5 -- this because the data represented on</p> <p>6 the exhibit is not reflective of the bar graphs</p> <p>7 that are in the published manuscript.</p> <p>8 So if you can just point us to where in</p> <p>9 the published manuscript you're pulling this</p> <p>10 from.</p> <p>11 MS. THOMPSON:</p> <p>12 All right.</p> <p>13 A This is -- the entire ordinate has</p> <p>14 changed. This is 25. This is 100.</p> <p>15 MS. THOMPSON:</p> <p>16 Q This is -- this is, from the chart,</p> <p>17 this is Figure 1. The color came out a little</p> <p>18 bit differently in the printing process,</p> <p>19 but the --</p> <p>20 MS. CURRY:</p> <p>21 This is not Figure 1.</p> <p>22 A No. Not even close. This is, in fact,</p> <p>23 Figure 3.</p> <p>24 MS. THOMPSON:</p>

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<p style="text-align: right;">Page 370</p> <p>1 Q PCR, CAT, SOD3. CAT activity and SOD</p> <p>2 activity.</p> <p>3 MS. THOMPSON:</p> <p>4 Are y'all looking? Mine are identical.</p> <p>5 Can you be --</p> <p>6 MS. CURRY:</p> <p>7 On the published manuscript, this chart</p> <p>8 does not represent --</p> <p>9 MS. THOMPSON:</p> <p>10 To Figure 1?</p> <p>11 MS. CURRY:</p> <p>12 -- to Figure 1.</p> <p>13 MS. THOMPSON:</p> <p>14 Let's go off the record.</p> <p>15 VIDEOGRAPHER:</p> <p>16 Going off the record at 4:55.</p> <p>17 (OFF THE RECORD.)</p> <p>18 VIDEOGRAPHER:</p> <p>19 We're back on the record at 4:59 p.m.</p> <p>20 MS. THOMPSON:</p> <p>21 Q Okay. Now that we've got that</p> <p>22 straightened out, so you'll agree that this is</p> <p>23 the -- the chart that shows the expression of</p> <p>24 antioxidant CAT and SKOV3 and the activity of the</p>	<p style="text-align: right;">Page 372</p> <p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A It could change them considerably,</p> <p>4 yeah.</p> <p>5 MS. THOMPSON:</p> <p>6 Q Do you want to change that to a</p> <p>7 question mark, or do you want to change that to</p> <p>8 no, they're not accurate?</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A Question mark will be fine.</p> <p>12 MS. THOMPSON:</p> <p>13 Q And that would go for all cell lines?</p> <p>14 A Well, the technology -- the techniques</p> <p>15 used was applied to all of them.</p> <p>16 MS. CURRY:</p> <p>17 Just so I know what we're doing here --</p> <p>18 I'm sorry -- is when you're saying results</p> <p>19 accurate in these four pictures, are -- are you</p> <p>20 talking about -- like is that based on raw data</p> <p>21 that's supposed to be in here? I'm just not sure</p> <p>22 what we're doing.</p> <p>23 MS. THOMPSON:</p> <p>24 These graphs are from the raw data.</p>
<p style="text-align: right;">Page 371</p> <p>1 same; correct?</p> <p>2 A You're on Figure 1?</p> <p>3 Q I am on Figure 1, yes.</p> <p>4 A Yeah. That's CAT and SKOV3?</p> <p>5 Q Yeah.</p> <p>6 A Yep.</p> <p>7 Q And we -- we are going through each</p> <p>8 cell line. The first column was Results</p> <p>9 Accurate, and I think --</p> <p>10 A So let me -- let me revise that.</p> <p>11 Q Okay.</p> <p>12 A Because now I understand what we're</p> <p>13 looking at.</p> <p>14 So I think there's a serious problem in</p> <p>15 the PCR, or at least I'd be concerned by that.</p> <p>16 These PCR MRNA levels were normalized to beta</p> <p>17 actin. And I think most of us would accept that</p> <p>18 using one housekeeping gene is not acceptable. I</p> <p>19 would expect at least two or three to make sure</p> <p>20 that there isn't a change in the stability of</p> <p>21 beta actin, which would throw off your</p> <p>22 quantification levels of those genes.</p> <p>23 Q And do you think that would render</p> <p>24 these results inaccurate?</p>	<p style="text-align: right;">Page 373</p> <p>1 MS. CURRY:</p> <p>2 But the raw data, we don't have. That</p> <p>3 hasn't --</p> <p>4 MS. THOMPSON:</p> <p>5 You've seen the raw data in the lab</p> <p>6 notebooks and Dr. Saed has -- is this an</p> <p>7 objection or is this --</p> <p>8 MS. CURRY:</p> <p>9 It's an object- -- I'm just honestly --</p> <p>10 I'm trying -- you're trying to have him create an</p> <p>11 exhibit --</p> <p>12 MS. THOMPSON:</p> <p>13 That's a speaking objection.</p> <p>14 MS. CURRY:</p> <p>15 -- and I'm trying to find out --</p> <p>16 MS. THOMPSON:</p> <p>17 If he understands it, it doesn't really</p> <p>18 matter whether you do or not, Dawn. I mean --</p> <p>19 MS. CURRY:</p> <p>20 And that's fine if you don't want an</p> <p>21 accurate record. That's fine.</p> <p>22 MS. THOMPSON:</p> <p>23 And he hasn't expressed that he doesn't</p> <p>24 understand.</p>

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<p>1 MS. CURRY: 2 That's fine. 3 MS. THOMPSON: 4 Q Dr. Birrer, do you understand what I'm 5 asking with this chart? If not, I'll explain it. 6 A Well, I -- I think -- it's a little bit 7 like the exercise this morning, which is we're 8 creating a document without all the information. 9 I don't have the raw data here. I mean, yeah, 10 it's in the notebooks, I suppose, somewhere. 11 Q And -- and you'll agree that these 12 charts are generated from raw data by a software 13 program. Correct? 14 And Dr. Saed testified to that. 15 Correct? 16 MS. CURRY: 17 Object to the form. 18 A Well, again, depending on what data's 19 put in -- 20 MS. THOMPSON: 21 Q Okay. 22 A -- you could get completely different 23 results. 24 Q I understand. But we're gonna look at</p>	<p>1 A Well, I think the -- if you're gonna 2 call them normal, then the normal primary -- the 3 human primary normal ovarian cell lines would be 4 more relevant. 5 MS. THOMPSON: 6 Q More relevant? But either one would be 7 relevant. Is that what you're saying? 8 MS. CURRY: 9 Object to form. 10 A No. I think the immortalized one is 11 not normal, so it wouldn't be relevant. 12 MS. THOMPSON: 13 Q Okay. So we'll make another column. 14 Well, we don't -- the immortalized and 15 the normal. 16 So the immortalized would be not 17 relevant? 18 A Right. 19 Q And the -- 20 A Yes. 21 Q Maybe I should get a clean -- let's -- 22 let's start over this chart. That's okay. I'll 23 make the next one neater. 24 Okay. Let's start again. And we're</p>
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<p>1 the data that was in the peer-reviewed published 2 paper. Okay? 3 Are the results relevant? And we can 4 go by each cell line. 5 MS. CURRY: 6 Object to the form. 7 MS. THOMPSON: 8 Q And yes or no or you don't know. 9 MS. CURRY: 10 Object to the form. 11 A Well, one of the challenges in this 12 paper is the purpose of the EL1 cell line. I 13 don't think those results are relevant. 14 MS. THOMPSON: 15 Q Okay. The other lines? 16 A The normal ovary, I would assume -- is 17 that primary cells? Right? We reviewed that? 18 Let me go back. 19 So I don't know if that's -- I don't 20 know if that's the HOS cell line or the -- the 21 ones from Cell Biologics. 22 Q Is one relevant and one not? 23 MS. CURRY: 24 Object to the form.</p>	<p>1 gonna distinguish between -- 2 A Uh-huh. 3 Q -- the immortalized, which is IM on the 4 chart, and that's going to be not relevant; 5 right? 6 A Correct. 7 Q And the normal cells are relevant, in 8 your mind? 9 A Uh-huh. 10 Q How about the fallopian tube, the FT33? 11 A Yeah. So that's immortalized also, so 12 I don't think it's particularly relevant. 13 Q Is it not relevant? 14 MS. CURRY: 15 Object to the form. 16 A Uh-huh. 17 MS. THOMPSON: 18 Q And that's because it's immortalized? 19 A Uh-huh. 20 Q Okay. And 3, cancer cell lines? 21 A So this is -- 22 MS. CURRY: 23 Object to the form. 24 A So this was a big -- this was a concern</p>

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<p style="text-align: right;">Page 378</p> <p>1 in the paper, which is that, as you know, SKOV3</p> <p>2 is a clear cell; we've got an endometrioid; and</p> <p>3 we don't even know where 2780 comes from, so I</p> <p>4 don't think they're relevant.</p> <p>5 MS. THOMPSON:</p> <p>6 Q And that's because of lacking a clear</p> <p>7 histologic relationship?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A That's right.</p> <p>11 MS. THOMPSON:</p> <p>12 Q Do those results show a biological</p> <p>13 effect from talcum powder?</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 A So I don't view that -- I don't -- I</p> <p>17 guess the answer is -- biologic effects?</p> <p>18 MS. THOMPSON:</p> <p>19 Q Does something happen when you put the</p> <p>20 baby powder in the cell culture?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 MS. THOMPSON:</p> <p>24 Q This is not related to whether you</p>	<p style="text-align: right;">Page 380</p> <p>1 Q As long as you approve of my work, we</p> <p>2 can -- we can switch the exhibit over to the one</p> <p>3 I'm doing.</p> <p>4 A Uh-huh.</p> <p>5 Q If the results are accurate, do they</p> <p>6 demonstrate a dose-dependent response?</p> <p>7 MS. CURRY:</p> <p>8 I object to the entirety of the</p> <p>9 exercise --</p> <p>10 MS. THOMPSON:</p> <p>11 Okay. You're --</p> <p>12 MS. CURRY:</p> <p>13 -- but I am following you in terms of</p> <p>14 the accuracy of you putting his answers down on</p> <p>15 the paper.</p> <p>16 MS. THOMPSON:</p> <p>17 Okay. All right. And we'll have the</p> <p>18 record, too.</p> <p>19 MS. THOMPSON:</p> <p>20 Q Do the answers show a dose-dependent</p> <p>21 response?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A So it depends on the cell line, I</p>
<p style="text-align: right;">Page 379</p> <p>1 agree with how it was, the dosage, whether the</p> <p>2 results are accurate or not.</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 A Yeah. It's really hard to interpret</p> <p>6 this because, again, I believe he used a control</p> <p>7 with DMSO. DMSO has fairly dramatic effects and</p> <p>8 he's not controlling for it. So, you know, I</p> <p>9 would say no.</p> <p>10 MS. THOMPSON:</p> <p>11 Q No biologic effects?</p> <p>12 A No biologic effects.</p> <p>13 Q On any of the cell lines?</p> <p>14 A Correct. Unless you call PCR effect --</p> <p>15 you know, PCR quantification biologic.</p> <p>16 Q Do you have your exhibit there?</p> <p>17 A Exhibit --</p> <p>18 Q Oh, well. We can -- we'll just use</p> <p>19 mine.</p> <p>20 A This one?</p> <p>21 Q I wondered if you wanted to be filling</p> <p>22 these in yourself. But as long as you correct</p> <p>23 my --</p> <p>24 A You go.</p>	<p style="text-align: right;">Page 381</p> <p>1 think. Right?</p> <p>2 MS. THOMPSON:</p> <p>3 Q Which cell line does not? So --</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 A If you look at the PCR, I don't know --</p> <p>7 and you look at everything but EL1, I don't know</p> <p>8 if those are statistically different. If you --</p> <p>9 if you pull it down, you can see it.</p> <p>10 MS. THOMPSON:</p> <p>11 Q Oh, sorry.</p> <p>12 A Yeah. See way on the top?</p> <p>13 Q If the paper says they were</p> <p>14 statistically significant, does that matter?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 A Well, it doesn't look like it to me.</p> <p>18 MS. THOMPSON:</p> <p>19 Q So are you gonna say no or you don't</p> <p>20 know?</p> <p>21 A No.</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 MS. THOMPSON:</p>

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<p style="text-align: right;">Page 382</p> <p>1 Q On all cell lines?</p> <p>2 A No. For EL1. Normal ovary.</p> <p>3 So, actually, for -- for -- what is</p> <p>4 that? That's B, SKOV3. So for SKOV3, it looks</p> <p>5 like nothing. It's -- from the mRNA level, it's</p> <p>6 all suppressed. It's all very low. I don't</p> <p>7 see -- I don't see -- if there's a P-value there,</p> <p>8 what is it between? The control and the 5? The</p> <p>9 control and the 20? The 20 and the 100? I don't</p> <p>10 know.</p> <p>11 The ELISA looks like -- this is for</p> <p>12 SKOV3; right? The ELISA looks like there's no</p> <p>13 effect until you get to 20 or 100.</p> <p>14 Q And you're eyeballing the statistical</p> <p>15 significance of these charts?</p> <p>16 A Well, that's why they --</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A That's why they put arrow bars in</p> <p>20 there.</p> <p>21 MS. THOMPSON:</p> <p>22 Q So reading Dr. Saed's results in the</p> <p>23 manuscript --</p> <p>24 A Uh-huh.</p>	<p style="text-align: right;">Page 384</p> <p>1 Q Well, you had the raw data to review,</p> <p>2 didn't you?</p> <p>3 MS. CURRY:</p> <p>4 Object to the form.</p> <p>5 MS. THOMPSON:</p> <p>6 Q It's on your materials considered list.</p> <p>7 A Well, his notebooks were very difficult</p> <p>8 to interpret.</p> <p>9 Q All the raw data was in his notebooks.</p> <p>10 If it -- if you are saying these results were not</p> <p>11 accurate, could you have looked it up in the lab</p> <p>12 notebooks?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A Yeah, I don't know. I'd have to go</p> <p>16 back and look at it. There were --</p> <p>17 MS. THOMPSON:</p> <p>18 Q Did you do that?</p> <p>19 MS. CURRY:</p> <p>20 Object to the form.</p> <p>21 A I looked at his notebooks. They were</p> <p>22 extremely hard to follow.</p> <p>23 MS. THOMPSON:</p> <p>24 Q Did you ask someone --</p>
<p style="text-align: right;">Page 383</p> <p>1 Q -- the CAT and SKOV -- this is Figure</p> <p>2 1 -- "mRNA and protein levels were significantly</p> <p>3 in a dose-dependent manner in talc-treated cells</p> <p>4 compared to controls."</p> <p>5 Do you disagree with Dr. Saed's</p> <p>6 analysis?</p> <p>7 A I disagree with that statement.</p> <p>8 Q So you're going to say, regardless of</p> <p>9 Dr. Saed's peer-reviewed conclusion, your</p> <p>10 opinion, these do not show a dose-dependent</p> <p>11 response --</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 MS. THOMPSON:</p> <p>15 Q -- based on your eyeballing of the</p> <p>16 chart?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form. That's --</p> <p>19 A Well, that -- I disagree with that</p> <p>20 statement. That implies that these are all</p> <p>21 statistically significant, and I can't imagine</p> <p>22 that's true, given the arrow bars. But it would</p> <p>23 be very helpful to have the raw data.</p> <p>24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 385</p> <p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 MS. THOMPSON:</p> <p>4 Q -- to get information? Because what's</p> <p>5 your evidence that the data wasn't included in</p> <p>6 the lab notebooks?</p> <p>7 MS. CURRY:</p> <p>8 Object to the form.</p> <p>9 A Well, I -- again, his notebooks were</p> <p>10 very poorly organized. There were things that</p> <p>11 were whited out. So it was hard to follow.</p> <p>12 MS. THOMPSON:</p> <p>13 Q Okay. What was whited out? Seriously.</p> <p>14 Was there any data whited out?</p> <p>15 MS. CURRY:</p> <p>16 Object to the form.</p> <p>17 MS. THOMPSON:</p> <p>18 Q You're making --</p> <p>19 A Well, do you have them here?</p> <p>20 MS. THOMPSON:</p> <p>21 Q I do.</p> <p>22 MS. CURRY:</p> <p>23 And the deposition transcript?</p> <p>24 MS. THOMPSON:</p>

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<p style="text-align: right;">Page 386</p> <p>1 I need the lab notebooks. Let's just 2 answer this, and I think we're going to move on 3 to something else. 4 Q In your opinion, are the results 5 dose-deponent? 6 MS. CURRY: 7 Object to the form. 8 A So I -- I guess the way to handle that 9 would be for -- there looks like there's a dose 10 dependency for some of the cell lines in certain 11 conditions but not all of them. Is that fair to 12 say? 13 MS. THOMPSON: 14 Q Well, so you don't believe 15 Dr. Saed's -- 16 A Conclusions. 17 Q -- conclusions? 18 A I don't agree with that one statement. 19 His statement is that basically all of the time 20 points demonstrated a dose-dependant effect of 21 talc. If that's true -- you can't see it here. 22 You see it in some. 23 Q Did -- did any of the peer-reviewers 24 raise a question about that statement?</p>	<p style="text-align: right;">Page 388</p> <p>1 publications using the same methodology and the 2 same assays? 3 MS. CURRY: 4 Object to the form. 5 A I didn't -- I didn't go through all of 6 his papers, no. 7 MS. THOMPSON: 8 Q Did you go through any of his previous 9 papers? 10 MS. CURRY: 11 Object to the form. 12 A I can't recall going through papers 13 that used this technology. 14 MS. THOMPSON: 15 Q But this technology has been 16 peer-reviewed and published -- 17 MS. CURRY: 18 Object to the form. 19 A Yes. 20 MS. THOMPSON: 21 Q -- previously? 22 And you're aware that Dr. Saed has 23 presented four abstracts based on this research; 24 correct?</p>
<p style="text-align: right;">Page 387</p> <p>1 A No. 2 Q And, in fact, the peer-reviewers said 3 his conclusions reflected the results; correct? 4 MS. CURRY: 5 Object to the form. 6 MS. THOMPSON: 7 Q The peer-reviewer that commented on it? 8 A The one reviewer. 9 Q The only one that commented on it? 10 A Yeah. 11 Q So are these question marks or which -- 12 which cell lines do you think are statistically 13 significant? 14 A Yeah. I think that's -- I think that's 15 probably reasonable, question marks. 16 Q Question marks on everything? 17 A Yeah. 18 Q And there's plenty of discussion for us 19 to go back and figure out the reasoning for that. 20 We may come back to the chart, but 21 there's some other things I want to cover, so 22 we'll -- we'll leave that with you disagreeing 23 with Dr. Saed's analysis. 24 Did you look at Dr. Saed's previous</p>	<p style="text-align: right;">Page 389</p> <p>1 A I believe so. 2 Q And abstracts are generally reviewed 3 prior to acceptance at a national meeting; 4 correct? 5 MS. CURRY: 6 Object to the form. 7 A Usually there's a program committee 8 that will review them. 9 MS. THOMPSON: 10 Q And would you agree that, generally, 11 four to six reviewers look at abstracts when 12 making the decision which to accept for a 13 meeting? 14 MS. CURRY: 15 Object to the form. 16 A It depends on the organization. But 17 there usually is -- it's certainly more than one 18 person. 19 MS. THOMPSON: 20 Q If -- if I told you Society For 21 Reproductive Investigation typically has four to 22 six reviewers and SGO has four to five reviewers 23 for each abstract, does that sound reasonable? 24 MS. CURRY:</p>

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<p style="text-align: right;">Page 390</p> <p>1 Object to the form.</p> <p>2 A You know, I think for the first</p> <p>3 society, the former one, I'm not familiar with</p> <p>4 them, but it sounds reasonable.</p> <p>5 SGO, I've been on the program</p> <p>6 committee. Sometimes it's a little less than</p> <p>7 that depending on how many abstracts you get.</p> <p>8 MS. THOMPSON:</p> <p>9 Q At least for this year, there were four</p> <p>10 to five reviewers, and the abstracts were scored</p> <p>11 numerically.</p> <p>12 Are you familiar with that system?</p> <p>13 MS. CURRY:</p> <p>14 Object to the form.</p> <p>15 A I am.</p> <p>16 MS. THOMPSON:</p> <p>17 Q And the -- and the top scoring</p> <p>18 abstracts were accepted for presentation?</p> <p>19 A Usually they'll put a cutoff on it,</p> <p>20 yeah.</p> <p>21 Q And in the two criteria that SGO</p> <p>22 reviewers looked at were, one, scientific</p> <p>23 validity; and two, clinical relevance.</p> <p>24 Does that sound right?</p>	<p style="text-align: right;">Page 392</p> <p>1 You would agree with me that there have</p> <p>2 been at least 20 to 30 eyes on this research;</p> <p>3 correct?</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 MS. THOMPSON:</p> <p>7 Q In various levels of review.</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A 20 to 30 sounds a little excessive but</p> <p>11 probably --</p> <p>12 MS. THOMPSON:</p> <p>13 Q Well, four abstracts, four to five</p> <p>14 reviewers each --</p> <p>15 A Oh, you're saying all of it?</p> <p>16 Q Yeah. Combined.</p> <p>17 MS. CURRY:</p> <p>18 Objection.</p> <p>19 MS. THOMPSON:</p> <p>20 Q Would you agree that there have been at</p> <p>21 least 25 eyes on this research?</p> <p>22 A Uh-huh. Some could have overlapped.</p> <p>23 MS. GARBER:</p> <p>24 Or 50 eyes, since there's two.</p>
<p style="text-align: right;">Page 391</p> <p>1 MS. CURRY:</p> <p>2 Object to the form.</p> <p>3 A That, I don't know.</p> <p>4 MS. THOMPSON:</p> <p>5 Q And -- and you'll agree that the</p> <p>6 mutation, the SNP data, was presented as a poster</p> <p>7 at this year's SGO meeting; correct?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A I didn't -- I didn't go to that poster,</p> <p>11 so I don't know what was on it. If it was a --</p> <p>12 if it was similar to the paper, I would assume</p> <p>13 so.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Okay. So if you have the manuscript</p> <p>16 that was reviewed by at least two reviewers and</p> <p>17 the editors of Gynecologic Oncology, you have the</p> <p>18 manuscript that was reviewed by at least one</p> <p>19 editor -- one reviewer and editor for</p> <p>20 Reproductive Sciences. You have abstracts that</p> <p>21 are each reviewed by four to five reviewers. He</p> <p>22 also has a book chapter that was reviewed,</p> <p>23 peer-reviewed by editors which included this</p> <p>24 data.</p>	<p style="text-align: right;">Page 393</p> <p>1 MS. THOMPSON:</p> <p>2 Q Fifty eyes.</p> <p>3 Are you aware of any other reviewers</p> <p>4 that raised the serious concerns that you seem to</p> <p>5 have with Dr. Saed's paper --</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 MS. THOMPSON:</p> <p>9 Q -- and -- and research?</p> <p>10 A I don't know any of the reviewers for</p> <p>11 the abstracts or the SGO. That's all kept</p> <p>12 confidential. So none of them have -- I haven't</p> <p>13 any firsthand knowledge that they said to me.</p> <p>14 But the review process hasn't raised -- hasn't</p> <p>15 necessarily raised the issues that I've raised.</p> <p>16 Q Okay.</p> <p>17 A But that doesn't change my opinion.</p> <p>18 Q I didn't ask you, actually. If it did,</p> <p>19 I didn't expect it to.</p> <p>20 I want to go through -- oh.</p> <p>21 (DEPOSITION EXHIBIT NUMBER 32 WAS</p> <p>22 MARKED FOR IDENTIFICATION.)</p> <p>23 MS. THOMPSON:</p> <p>24 Q And did you -- did you review</p>

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<p>1 Dr. Saed's review article published in 2 Gynecologic Oncology in 2017? 3 A I think I saw this. Is this on 4 oxidative stress? 5 Q Yes. 6 A Yeah. Yeah. 7 Q And -- and do you know if this review 8 article was invited or submitted and 9 peer-reviewed in the process? 10 A I don't know. 11 Q But, as you've testified before, and 12 typically authors of review articles in reputable 13 journals are felt to be experts in the field; 14 correct? 15 MS. CURRY: 16 Object to the form. 17 A They generally are. 18 MS. THOMPSON: 19 Q And -- 20 MS. CURRY: 21 Did you mark this as an exhibit? 22 MS. EVERETT: 23 It's Exhibit 32. 24 MS. THOMPSON:</p>	<p>1 MS. THOMPSON: 2 Q Yes. 3 A It's not the same phrase. Essential 4 role -- actually, the essential role here is 5 pretty narrow. But it -- but, you know, I 6 wouldn't quibble about that. It's in the same 7 range. 8 Q It's a similar concept that's -- that 9 was published in the review article; correct? 10 A Uh-huh. 11 MS. CURRY: 12 Object to the form. 13 MS. THOMPSON: 14 Q Reading the abstract "Clinical and 15 epidemiological investigations have provided 16 evidence supporting the role of reactive oxygen 17 species, ROS, and reactive nitrogen species, RNS, 18 collectively known as oxidative stress in the 19 etiology of cancer." 20 Would you agree with that statement? 21 MS. CURRY: 22 Object to the form. 23 A Yep. 24 MS. THOMPSON:</p>
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<p>1 32. 2 MS. CURRY: 3 Okay. Thank you. 4 MS. THOMPSON: 5 Q And just looking at the abstract on -- 6 well, first on the highlights -- this review 7 article updates the role of oxidative stress and 8 the pathogenesis of ovarian cancer. 9 The first highlight is "Oxidative 10 Stress Plays an Essential Role in the 11 Pathogenesis of Ovarian Cancer." 12 A Where are you? I'm sorry. 13 Q The highlights at the top. 14 A Oh. The bullet points? 15 Q Bullet point, highlights. 16 A Okay. 17 Q And you'll agree that -- that statement 18 is essentially the same as the one in the talcum 19 powder article that was asked to be removed 20 because of the data not supporting that 21 statement; correct? 22 MS. CURRY: 23 Object to the form. 24 A You're going on submission?</p>	<p>1 Q "Exogenous factors such as chronic 2 inflammation, infection and hypoxia are major 3 sources of cellular oxidative stress." 4 Would you agree with that statement? 5 MS. CURRY: 6 Object to the form. 7 A Well, I would just refine it to say 8 they were sources. I don't know if they're the 9 major sources. In certain conditions there may 10 be other sources. So it's a little bit of a 11 generality. 12 MS. THOMPSON: 13 Q "Specifically oxidative stress plays an 14 important role in the pathogenesis, 15 neoangiogenesis and dissemination of local or 16 distant ovarian cancer, as it is known to induce 17 phenotypic modifications of tumor cells by 18 crosstalk between tumor cells and the surrounding 19 stroma." 20 Do you agree with that statement? 21 A Well, that's a mouthful. There's a lot 22 in there, and I'm not so sure I know exactly what 23 he's talking about. Pathogenesis is pretty 24 general. Blood vessel formation is a different</p>

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<p>1 process. So --</p> <p>2 Q But certainly the reviewers and the</p> <p>3 editors of the journal, when they published the</p> <p>4 review article --</p> <p>5 A Uh-huh.</p> <p>6 Q -- thought that was accurate</p> <p>7 information; correct?</p> <p>8 A They did.</p> <p>9 MS. CURRY:</p> <p>10 Object to the form.</p> <p>11 A Yeah.</p> <p>12 MS. THOMPSON:</p> <p>13 Q Going to Table 1 on page 598, that's a</p> <p>14 "Summary of the Oxidant and Antioxidant</p> <p>15 Expression and Sensitive and Chemoresistant</p> <p>16 Ovarian Cancer." You'll agree that these were</p> <p>17 essentially the same markers that Dr. Saed</p> <p>18 studied in the talcum powder experiments;</p> <p>19 correct?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 MS. THOMPSON:</p> <p>23 Q NPO, INOS?</p> <p>24 A I think so. I think so. I'm just</p>	<p>1 MS. THOMPSON:</p> <p>2 Q But the -- but the markers are the</p> <p>3 same, essentially?</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 A The markers are the same.</p> <p>7 MS. THOMPSON:</p> <p>8 Q And they're published in this review</p> <p>9 article, correct, in Gynecologic Oncology?</p> <p>10 A They're reported here and published.</p> <p>11 Q And you'll agree there have been some</p> <p>12 other molecular studies relating to talcum powder</p> <p>13 and cell culture; correct?</p> <p>14 MS. CURRY:</p> <p>15 Object to the form.</p> <p>16 A I believe so.</p> <p>17 MS. THOMPSON:</p> <p>18 Q Are you familiar with a Shukla paper?</p> <p>19 A Yes, I am.</p> <p>20 Q I'll mark the Shukla paper Exhibit 33.</p> <p>21 (DEPOSITION EXHIBIT NUMBER 33 WAS</p> <p>22 MARKED FOR IDENTIFICATION.)</p> <p>23 MS. THOMPSON:</p> <p>24 Q Okay. And this paper was published in</p>
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<p>1 checking all of them. Did they --</p> <p>2 Q And generally speaking.</p> <p>3 A Certainly the lower list is all in</p> <p>4 there, yeah.</p> <p>5 Q So -- so these -- these oxidants,</p> <p>6 antioxidants that Dr. Saed studied with the</p> <p>7 talcum powder, he had published before; correct?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Well, this is a review article. He's</p> <p>11 not publishing primary data right now. He's just</p> <p>12 noting it.</p> <p>13 MS. THOMPSON:</p> <p>14 Q A review article noting the relevance</p> <p>15 of those assays for oxidative stress in ovarian</p> <p>16 cancer; correct?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A Well, again, I'm refining that a little</p> <p>20 bit because this table really looks for</p> <p>21 expression comparing standard ovarian cancer to</p> <p>22 chemoresistance. That's really not what this</p> <p>23 paper is about. So it's kind of apples and</p> <p>24 oranges.</p>	<p>1 2008; correct?</p> <p>2 MS. CURRY:</p> <p>3 Object to the form.</p> <p>4 MS. THOMPSON:</p> <p>5 Q Sorry. Received in --</p> <p>6 A That was in '9.</p> <p>7 Q In formal form, 2008.</p> <p>8 MS. CURRY:</p> <p>9 Do you have a copy?</p> <p>10 A This is in 2009, I have it.</p> <p>11 MS. THOMPSON:</p> <p>12 Q The title is "Alterations in Gene</p> <p>13 Expression in Human Mesothelia Cells Correlate</p> <p>14 with Mineral Pathogenicity."</p> <p>15 Is that the title of this paper that</p> <p>16 you have?</p> <p>17 A Yes. Yes.</p> <p>18 Q Okay. And it was published in --</p> <p>19 A I have it 2009.</p> <p>20 Q Oh. No. We're looking at -- I'm</p> <p>21 looking at that received in final form, and</p> <p>22 you're -- when it actually appeared. You're</p> <p>23 correct. 2009.</p> <p>24 And this paper looked at cell culture</p>

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<p style="text-align: right;">Page 402</p> <p>1 with asbestos applied; correct?</p> <p>2 A This looked at asbestos, nonfibrous</p> <p>3 talc, and titanium dioxide.</p> <p>4 Q Correct.</p> <p>5 A Or glass beads.</p> <p>6 Q And if you'll turn to Table 2, it</p> <p>7 reports on gene expression and mesothelial cells</p> <p>8 at low and high doses at 8 and 24 hours for the</p> <p>9 low dose and 8 hours for the high dose. Correct?</p> <p>10 A This is genes that are affected by</p> <p>11 asbestos.</p> <p>12 Q Correct.</p> <p>13 And, then, if you'll look at table --</p> <p>14 A And this -- sorry.</p> <p>15 Q -- Table 3, which are the genes</p> <p>16 upregulated by nonfibrous talc, you'll see that</p> <p>17 testing was done at 8 hours at low and high dose.</p> <p>18 And it appears that there was no testing done at</p> <p>19 24 hours for talc.</p> <p>20 Is that your understanding?</p> <p>21 A I believe so.</p> <p>22 Q And, yet, there --</p> <p>23 A I'm sorry. Can I refine that?</p> <p>24 MS. CURRY:</p>	<p style="text-align: right;">Page 404</p> <p>1 Q Yeah, ATF.</p> <p>2 And those are cancer genes; correct?</p> <p>3 Or genes affiliated -- associated with cancer?</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 A Well, a lot of genes are.</p> <p>7 ATF3 --</p> <p>8 MS. THOMPSON:</p> <p>9 Q ATF3 and interleukin 8 are often</p> <p>10 studied in relationship to cancer association;</p> <p>11 correct?</p> <p>12 MS. CURRY:</p> <p>13 Object to the form.</p> <p>14 A I'd say interleukin 8. I don't -- I</p> <p>15 know of less data for ATF3. It's a transcription</p> <p>16 factor, so I don't know the story there.</p> <p>17 But your original question, these are</p> <p>18 statistically significant increases at 8 hours</p> <p>19 for talc; right?</p> <p>20 MS. THOMPSON:</p> <p>21 Q And 24 hours for talc was not</p> <p>22 performed; correct?</p> <p>23 MS. CURRY:</p> <p>24 Object to the form.</p>
<p style="text-align: right;">Page 403</p> <p>1 Object to the form. Sorry.</p> <p>2 A They were -- it was checked but the</p> <p>3 changes were not observed.</p> <p>4 MS. THOMPSON:</p> <p>5 Q Where do you see that?</p> <p>6 A Well, that may be -- hang on. "These</p> <p>7 are mesothelial cells..." Yeah. Right --</p> <p>8 assuming I'm reading this right.</p> <p>9 Right below the table it says "...were</p> <p>10 initially -- were observed initially with talc at</p> <p>11 8 hours. However, these changes were not</p> <p>12 observed at 24 hours. Suggesting that the human</p> <p>13 mesothelial cells adapt to this mineral."</p> <p>14 Q If you'll look at Table -- at Figure</p> <p>15 4 --</p> <p>16 A Figure 4.</p> <p>17 Q -- you do see that there are</p> <p>18 significant increases in both nonfibrous talc and</p> <p>19 the crocidolite asbestos; correct?</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 A So this is quantitative PCR of two</p> <p>23 genes; right? This is ATF3?</p> <p>24 MS. THOMPSON:</p>	<p style="text-align: right;">Page 405</p> <p>1 A It was performed but they didn't see</p> <p>2 any changes.</p> <p>3 MS. THOMPSON:</p> <p>4 Q Was it performed at the high dose?</p> <p>5 A Well, let's see. I can't answer that.</p> <p>6 It may be buried in here somewhere. I do -- I do</p> <p>7 note that in this paper they didn't detect a lot</p> <p>8 of gene changes with talc.</p> <p>9 Q They did detect gene changes with talc,</p> <p>10 did they not?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A Well, they didn't detect a lot. There</p> <p>14 were some.</p> <p>15 MS. THOMPSON:</p> <p>16 Q I didn't ask if there were a lot.</p> <p>17 There were gene changes with talc?</p> <p>18 A Uh-huh.</p> <p>19 Q Would you consider that a biological</p> <p>20 effect?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A So, I -- yeah. I don't consider it</p> <p>24 biologic. It may be transcriptional.</p>

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<p style="text-align: right;">Page 406</p> <p>1 MS. THOMPSON: 2 Q And you've looked at the Buz'Zard 3 paper; correct? The Pycnogenol paper, does that 4 sound familiar? 5 A Well, I don't recognize that name. 6 Yeah. I did look at it. 7 Q Okay. I'm gonna mark that as Exhibit 8 34. 9 (DEPOSITION EXHIBIT NUMBER 34 WAS 10 MARKED FOR IDENTIFICATION.) 11 MS. THOMPSON: 12 Q And you'll agree that this paper looked 13 at neoplastic transformation in humans' ovarian 14 cell cultures exposed to talc; correct? 15 A Well, this gets back to what we 16 discussed before. I think they -- they -- the 17 title says it and they -- and they argue that 18 what they've shown is transformation. I don't -- 19 I don't agree with that. 20 Q Well, at least the authors say that, in 21 reading from the abstract, two-thirds of the way 22 down, "Talc increased proliferation, induced 23 neoplastic transformation and increased ROS 24 generation timed dependently in the ovarian cells</p>	<p style="text-align: right;">Page 408</p> <p>1 think about Buz'Zard. I'd have to cross-compare 2 that. 3 MS. THOMPSON: 4 Q Well, I'm just asking you if it refutes 5 his findings. 6 MS. CURRY: 7 Object to the form. 8 A No. I -- I'm thinking about that. I 9 think his ROS generation is a little bit 10 different, Buz'Zard. 11 MS. THOMPSON: 12 Q The ROS generation may be a little bit 13 different, but it does show ROS generation in 14 that paper; correct? 15 MS. CURRY: 16 Object to the form. 17 A Now, the Buz'Zard was -- was, for lack 18 of a better term, bizarre, because there were 19 differential effects in terms of production of 20 ROS depending on the concentration. So I found 21 it very difficult. And the interpretation that 22 they had was, I thought, misleading. 23 MS. THOMPSON: 24 Q But the question was: Did it in any</p>
<p style="text-align: right;">Page 407</p> <p>1 and dosed dependently in the p.m." 2 And that's at least what the authors 3 conclude; right? 4 A That's what they say in the abstract, 5 yes. 6 Q And also conclude that "The data 7 suggests that talc may contribute to ovarian 8 neoplastic transformation" -- 9 A Where are you now? I'm sorry. The 10 next sentence? 11 Q Next-to-last sentence. 12 A Yep. 13 Q "The data suggests that talc may 14 contribute to ovarian neoplastic transformation 15 and Pyc reduced the talc-induced transformation." 16 That's what the authors concluded; 17 correct? 18 A That's what they say. 19 Q Do either the Shukla paper or the 20 Buz'Zard paper refute Dr. Saed's research 21 findings? 22 MS. CURRY: 23 Object to the form. 24 A I don't think Shukla does. I'd have to</p>	<p style="text-align: right;">Page 409</p> <p>1 way refute Dr. Saed's findings? 2 MS. CURRY: 3 Object to the form. 4 A In -- in terms of comparing this to 5 that? 6 MS. THOMPSON: 7 Q Yes. 8 A I'd have to take a close look at that. 9 It's not something I thought about. 10 Q Okay. But there's nothing that's 11 obvious that refutes Dr. Saed's -- 12 A It's not leaping out to me. 13 (DEPOSITION EXHIBIT NUMBER 35 WAS 14 MARKED FOR IDENTIFICATION.) 15 MS. THOMPSON: 16 Q Okay. I'm marking as Exhibit 35 a 17 paper by Akhtar from 2010. 18 Have you seen this paper? 19 A This one, I don't think I reviewed. 20 Let me just see if it's on my list. No. 21 Q And are you aware from Dr. Saed's 22 deposition that he referred to the -- this paper 23 to establish his dosages for the talc experiments 24 that Dr. Saed performed?</p>

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<p>1 A In terms of what he did?</p> <p>2 Q Yes.</p> <p>3 A No, I didn't. I'm not aware of that</p> <p>4 from his deposition.</p> <p>5 Q Looking at the paper --</p> <p>6 A Yeah.</p> <p>7 Q -- does that look reasonable?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A This is way out of my purview with iron</p> <p>11 mediated lipid peroxidase in A459 cells, which</p> <p>12 are lung cancer. I don't know the relevance of</p> <p>13 this to what we're addressing here.</p> <p>14 MS. THOMPSON:</p> <p>15 Q Well, let's read what he says --</p> <p>16 A Sure.</p> <p>17 Q -- in the abstract.</p> <p>18 "Talc particles, the basic ingredient</p> <p>19 in different kinds of talc-based cosmetic and</p> <p>20 pharmaceutical products pose a health risk to</p> <p>21 pulmonary and ovarian systems due to domestic and</p> <p>22 occupational exposures."</p> <p>23 Is that what the authors say?</p> <p>24 A Correct.</p>	<p>1 MS. THOMPSON:</p> <p>2 Q Well, it's the first statement of the</p> <p>3 abstract.</p> <p>4 A Right.</p> <p>5 Q Do you think that's just an irrelevant</p> <p>6 statement, that they put as the first -- the</p> <p>7 introductory sentence to their paper?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Well, I think that's their supposition.</p> <p>11 They make that statement. I get it. But that</p> <p>12 doesn't mean that this experiment is relevant to</p> <p>13 that.</p> <p>14 MS. THOMPSON:</p> <p>15 Q I'm asking do the authors think it was</p> <p>16 relevant?</p> <p>17 MS. CURRY:</p> <p>18 Object to the form.</p> <p>19 A You'd have to address it with them. I</p> <p>20 don't know.</p> <p>21 MS. THOMPSON:</p> <p>22 Q "The talc particles, the basic</p> <p>23 ingredient in different kinds of talc-based</p> <p>24 cosmetic and pharmaceutical products pose a</p>
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<p>1 Q So at least the authors thought that</p> <p>2 this experiment had relevance to talc-based</p> <p>3 cosmetic products; correct?</p> <p>4 MS. CURRY:</p> <p>5 Object to the form.</p> <p>6 A Yeah. I think it's in that sentence.</p> <p>7 MS. THOMPSON:</p> <p>8 Q And at least the authors thought that</p> <p>9 these experiments had relevance to the ovarian</p> <p>10 system; correct?</p> <p>11 MS. CURRY:</p> <p>12 Object to the form.</p> <p>13 A Well, they mentioned it. And as a -- I</p> <p>14 think as a premise to the experiment. That</p> <p>15 doesn't mean it's relevant.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Well, it's a -- you would assume that</p> <p>18 if it's a premise to do the experiment, that they</p> <p>19 thought the experiments would be relevant to the</p> <p>20 question that they're asking; correct?</p> <p>21 MS. CURRY:</p> <p>22 Object to the form.</p> <p>23 A There's no question there. That's a</p> <p>24 statement. It's in the --</p>	<p>1 health risk to pulmonary and ovarian systems due</p> <p>2 to domestic and occupational exposure."</p> <p>3 And then they go on to why they're</p> <p>4 studying talc particles.</p> <p>5 Is -- is it your testimony that you</p> <p>6 don't know whether the authors thought that was</p> <p>7 relevant or not?</p> <p>8 MS. CURRY:</p> <p>9 Object to the form.</p> <p>10 A Well, it's speculation. I don't know</p> <p>11 what was in their mind. I can read this. I see</p> <p>12 what they did. And that opening statement is,</p> <p>13 again, sort of setting the -- setting the plate.</p> <p>14 But is this system relevant to that? I don't</p> <p>15 know. Lipid peroxidation --</p> <p>16 MS. THOMPSON:</p> <p>17 Q But -- but you would agree that the</p> <p>18 peer-reviewers and the editors of this journal</p> <p>19 accepted this paper with the introduction that</p> <p>20 talc particles posed a risk to pulmonary and</p> <p>21 ovarian systems and that the investigators at</p> <p>22 least did the experiments and published the</p> <p>23 paper; correct?</p> <p>24 MS. CURRY:</p>

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<p style="text-align: right;">Page 414</p> <p>1 Object to the form.</p> <p>2 A Did the work and published the paper.</p> <p>3 Agree.</p> <p>4 MS. THOMPSON:</p> <p>5 Q And in the conclusion, the authors</p> <p>6 state "We have presented a preliminary data on</p> <p>7 the toxicity response elicited by the two types</p> <p>8 of talc nano particles depending on their</p> <p>9 different geologic origin," and then go on to</p> <p>10 conclude, the end, "Data clearly suggests that</p> <p>11 exposure to talc, particularly nanopowder, should</p> <p>12 be protected in humans at risk of occupational as</p> <p>13 well as domestic exposure."</p> <p>14 That's the conclusions of the authors</p> <p>15 based on this research; correct?</p> <p>16 A That's the last sentence? Is that the</p> <p>17 last sentence?</p> <p>18 Q Yes.</p> <p>19 A Yeah. That's what they say.</p> <p>20 Q That is in the conclusion?</p> <p>21 A That's what they say.</p> <p>22 Q And that is the "Conclusion" section of</p> <p>23 the paper; correct?</p> <p>24 A Correct.</p>	<p style="text-align: right;">Page 416</p> <p>1 Object to the form.</p> <p>2 A Well, I just saw it. I haven't</p> <p>3 reviewed it. I would be concerned that they're</p> <p>4 in a completely different cell system. And, as</p> <p>5 you know, there's just huge differences in tissue</p> <p>6 responses.</p> <p>7 MS. THOMPSON:</p> <p>8 Q Would that automatically make it</p> <p>9 irrelevant, in your mind?</p> <p>10 MS. CURRY:</p> <p>11 Object to the form.</p> <p>12 A I would -- I'd like to read the paper.</p> <p>13 But I'd be concerned. I would start out with a</p> <p>14 certain concern about that and then go through</p> <p>15 the paper.</p> <p>16 MS. THOMPSON:</p> <p>17 Q Okay. We can go off the record, and</p> <p>18 you -- you can look at the paper.</p> <p>19 A Okay.</p> <p>20 VIDEOGRAPHER:</p> <p>21 Off the record at 5:38 p.m.</p> <p>22 (OFF THE RECORD.)</p> <p>23 VIDEOGRAPHER:</p> <p>24 We're back on the record at 5:40 p.m.</p>
<p style="text-align: right;">Page 415</p> <p>1 (DEPOSITION EXHIBIT NUMBER 36 WAS</p> <p>2 MARKED FOR IDENTIFICATION.)</p> <p>3 MS. THOMPSON:</p> <p>4 Q I'm marking as Exhibit 36 another paper</p> <p>5 by Akhtar and colleagues published in 2012.</p> <p>6 Have you seen that paper, Dr. Birrer?</p> <p>7 A No.</p> <p>8 Q This paper is titled "Cytotoxicity and</p> <p>9 Apoptosis" --</p> <p>10 MS. CURRY:</p> <p>11 Do you have a copy? Sorry.</p> <p>12 MS. THOMPSON:</p> <p>13 I'm sorry.</p> <p>14 MS. CURRY:</p> <p>15 Thank you.</p> <p>16 MS. THOMPSON:</p> <p>17 Q This paper is titled "Cytotoxicity and</p> <p>18 Apoptosis Induction by Nano-Scale Talc Particles</p> <p>19 From Two Different Geographical Regions in Human</p> <p>20 Lung Epithelial Cells."</p> <p>21 Is it your opinion that this paper is</p> <p>22 irrelevant because it tested the biological</p> <p>23 effects of talc in human lung epithelial cells?</p> <p>24 MS. CURRY:</p>	<p style="text-align: right;">Page 417</p> <p>1 MS. THOMPSON:</p> <p>2 Q Dr. Birrer, this article titled</p> <p>3 "Cytotoxicity and Apoptosis Induction by</p> <p>4 Nano-Scale Talc Particles from Two Different</p> <p>5 Geographical Regions in Human Lung Epithelial</p> <p>6 Cells" is by the same authors of the paper we</p> <p>7 just discussed; right?</p> <p>8 A Correct. I don't know if they're all</p> <p>9 on here, but it's the same group.</p> <p>10 Q Same group.</p> <p>11 A Yeah.</p> <p>12 Q Going to the last sentence on the first</p> <p>13 page in the introduction, the authors state:</p> <p>14 "Epidemiologic evidence also suggest a possible</p> <p>15 association between genital use of talcum powder</p> <p>16 and risk of ovarian cancer. Talc also appears to</p> <p>17 induce reactive oxygen, ROS, generation,</p> <p>18 oxidative stress, and inflammation."</p> <p>19 Is that what the authors state</p> <p>20 regarding the epidemiology of talcum powder and a</p> <p>21 reason for studying the cellular response?</p> <p>22 MS. CURRY:</p> <p>23 Object to the form.</p> <p>24 A So the first statement is about</p>

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<p style="text-align: right;">Page 418</p> <p>1 epidemiologic evidence. The second statement is 2 about reactive oxygen species. And they don't 3 say anything about why there's a reason to study. 4 They just make those statements. 5 MS. THOMPSON: 6 Q Is it your testimony that they would 7 just put -- put that statement in randomly in the 8 introduction to their paper about cytotoxicity and 9 apoptosis with talc particles? 10 MS. CURRY: 11 Object to the form. 12 A It wouldn't be random. But, again, I 13 think it's just a piece of information that this 14 has been studied before in a different system. 15 MS. THOMPSON: 16 Q And you would -- and they cite to 17 Buz'Zard, the paper we just reviewed; correct? 18 A Uh-huh. Yes. 19 Q And they start -- cite to Langseth; 20 correct? 21 A Yes. 22 Q And in previous testimony you have 23 testified that you think that Langseth is a -- is 24 a high-quality paper. Do you remember that?</p>	<p style="text-align: right;">Page 420</p> <p>1 Is that what the authors conclude from 2 the experiments that they did on nano talc 3 particles? 4 A That's what they say right there, yeah. 5 Q And we've established earlier that the 6 baby powder is a mixed particle-sized product; 7 correct? 8 MS. CURRY: 9 Object to the form. 10 A Well, we talked about talc particles, 11 and I simply said my understanding is not as a 12 mineralogist, but my understanding is a different 13 spectrum. I don't -- 14 MS. THOMPSON: 15 Q And do you know one way or the other 16 whether some of the particles in baby powder 17 could be classified as nano particles? 18 A No, I don't know that. 19 Q Do either of the Akhtar papers that we 20 just looked at refute Dr. Saed's research? 21 MS. CURRY: 22 Object to the form. 23 A The only comment I would make on that 24 is that this -- and again, I looked at this for</p>
<p style="text-align: right;">Page 419</p> <p>1 MS. CURRY: 2 Object to the form. 3 A Yeah. I'd have to see that. 4 MS. THOMPSON: 5 Q Okay. 6 A But I'm more familiar with Buz'Zard. 7 Q Okay. Well, we just looked at that 8 one; right? 9 But at least -- 10 A Yeah. 11 Q -- that's what the authors state in 12 their introduction -- 13 A Yeah. 14 Q -- regarding talc; correct? 15 A Yes. 16 Q And, then, we'll just go to the 17 conclusion. 18 A Uh-huh. 19 Q The last paragraph. "In conclusion, 20 both IN" -- which is Indian nano particles or 21 nano talc -- "and CN" -- which is commercial nano 22 talc particles, "significantly induce 23 cytotoxicity, oxidative stress and apoptosis in 24 human lung epithelial cells."</p>	<p style="text-align: right;">Page 421</p> <p>1 literally five minutes, but I went through some 2 of the figures. This paper shows a lot of 3 cytotoxicity and apoptosis with the effect of 4 talc. That's -- and this is actually in a cancer 5 cell line; right? It's human lung epithelial 6 cells. I don't think they're -- they're at least 7 immortalized. So that strikes me as different 8 than the proliferative effect he's describing. 9 MS. THOMPSON: 10 Q That wasn't my question. 11 A Okay. 12 Q My question: Do these results refute 13 Dr. Saed's work? 14 MS. CURRY: 15 Object to the form. 16 A Well, this is in lung cancer, so it's 17 pretty much irrelevant. 18 MS. THOMPSON: 19 Q And where -- where are you finding that 20 it's in lung cancer cells? 21 A Human lung epithelial A549 cells. I 22 worked with them quite a bit. It's a lung cancer 23 cell line. It's an adenocarcinoma. Top of page 24 396.</p>

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<p>1 Q Human lung epithelial cells?</p> <p>2 A Uh-huh.</p> <p>3 Q Those are cancer cells?</p> <p>4 A A549, if it's the same A549 which I</p> <p>5 know about, which I think it is, that's an</p> <p>6 adenocarcinoma.</p> <p>7 Q Do you see anywhere in the paper where</p> <p>8 it describes those as cancer cells?</p> <p>9 A Just let me look at the back. I don't</p> <p>10 see it, although I've rushed through this. But I</p> <p>11 don't see it.</p> <p>12 Q I know. I don't see it either.</p> <p>13 They're just described as human lung epithelial</p> <p>14 cells, which doesn't sound like they were</p> <p>15 considered to be cancer cells.</p> <p>16 I'm not sure I got the answer to the</p> <p>17 question "Is there anything in either of these</p> <p>18 Akhtar papers that refutes Dr. Saed's findings?"</p> <p>19 A No.</p> <p>20 MS. CURRY:</p> <p>21 Object to the form.</p> <p>22 MS. THOMPSON:</p> <p>23 Q Do both of these Akhtar papers</p> <p>24 demonstrate biological effect from talc particles</p>	<p>1 MS. CURRY:</p> <p>2 Oh. I'm so sorry. Thank you.</p> <p>3 EXAMINATION</p> <p>4 BY MS. CURRY:</p> <p>5 Q Dr. Birrer, you have reviewed</p> <p>6 Dr. Clarke-Pearson's expert report; correct?</p> <p>7 A Yes.</p> <p>8 Q Do you think his opinions overall are</p> <p>9 based on sound science?</p> <p>10 A No.</p> <p>11 Q Do you defer to him on any issue</p> <p>12 presented in this case?</p> <p>13 A No.</p> <p>14 Q Do you defer to any of the plaintiffs'</p> <p>15 experts on any issues presented in this case?</p> <p>16 A No.</p> <p>17 MS. CURRY:</p> <p>18 I have no further questions.</p> <p>19 Thank you.</p> <p>20 MS. THOMPSON:</p> <p>21 I'm done.</p> <p>22 VIDEOGRAPHER:</p> <p>23 Okay. This concludes this deposition.</p> <p>24 The time is 6:04 p.m. We're off the</p>
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<p>1 on cell culture --</p> <p>2 MS. CURRY:</p> <p>3 Object to --</p> <p>4 MS. THOMPSON:</p> <p>5 Q -- lines?</p> <p>6 MS. CURRY:</p> <p>7 Object to the form.</p> <p>8 A I would say yes, that there is some</p> <p>9 activity.</p> <p>10 MS. THOMPSON:</p> <p>11 If we can take just a short break, I</p> <p>12 think I'm finished.</p> <p>13 VIDEOGRAPHER:</p> <p>14 Off the record at 5:48 p.m.</p> <p>15 (OFF THE RECORD.)</p> <p>16 VIDEOGRAPHER:</p> <p>17 We're back on the record at 6:03 p.m.</p> <p>18 MS. THOMPSON:</p> <p>19 Dr. Birrer, I have no further</p> <p>20 questions. Thank you for your time today.</p> <p>21 MS. CURRY:</p> <p>22 I have just a few follow-up questions.</p> <p>23 VIDEOGRAPHER:</p> <p>24 Counsel, your microphone.</p>	<p>1 record.</p> <p>2 (Deposition concluded at 6:04 p.m.)</p> <p>3</p> <p>4</p> <p>5</p> <p>6</p> <p>7</p> <p>8</p> <p>9</p> <p>10</p> <p>11</p> <p>12</p> <p>13</p> <p>14</p> <p>15</p> <p>16</p> <p>17</p> <p>18</p> <p>19</p> <p>20</p> <p>21</p> <p>22</p> <p>23</p> <p>24</p>

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<p>1 CERTIFICATE</p> <p>2 STATE OF ALABAMA)</p> <p>3 COUNTY OF MOBILE)</p> <p>4</p> <p>5 I do hereby certify that the above and</p> <p>6 foregoing transcript of proceedings in the matter</p> <p>7 aforementioned was taken down by me in machine</p> <p>8 shorthand, and the questions and answers thereto</p> <p>9 were reduced to writing under my personal</p> <p>10 supervision, and that the foregoing represents a</p> <p>11 true and correct transcript of the proceedings</p> <p>12 given by said witness upon said hearing.</p> <p>13 I further certify that I am neither of</p> <p>14 counsel nor of kin to the parties to the action,</p> <p>15 nor am I in anywise interested in the result of</p> <p>16 said cause.</p> <p>17 Signed this 22nd day of March, 2019.</p> <p>18</p> <p>19</p> <p>20 LOIS ANNE ROBINSON, RDR</p> <p>21 COURT REPORTER, NOTARY PUBLIC</p> <p>22 STATE OF ALABAMA AT LARGE</p> <p>23 ACCR# 352; EXPIRES 9/30/19</p> <p>24</p>	<p>1 - - - - -</p> <p>2 E R R A T A</p> <p>3 - - - - -</p> <p>4 PAGE LINE CHANGE</p> <p>5</p> <p>6 REASON: _____</p> <p>7</p> <p>8 REASON: _____</p> <p>9</p> <p>10 REASON: _____</p> <p>11</p> <p>12 REASON: _____</p> <p>13</p> <p>14 REASON: _____</p> <p>15</p> <p>16 REASON: _____</p> <p>17</p> <p>18 REASON: _____</p> <p>19</p> <p>20 REASON: _____</p> <p>21</p> <p>22 REASON: _____</p> <p>23</p> <p>24 REASON: _____</p>
<p>Page 427</p> <p>1 INSTRUCTIONS TO WITNESS</p> <p>2</p> <p>3 Please read your deposition</p> <p>4 over carefully and make any necessary</p> <p>5 corrections. You should state the reason</p> <p>6 in the appropriate space on the errata</p> <p>7 sheet for any corrections that are made.</p> <p>8 After doing so, please sign</p> <p>9 the errata sheet and date it.</p> <p>10 You are signing same subject</p> <p>11 to the changes you have noted on the</p> <p>12 errata sheet, which will be attached to</p> <p>13 your deposition.</p> <p>14 It is imperative that you</p> <p>15 return the original errata sheet to the</p> <p>16 deposing attorney within thirty (30) days</p> <p>17 of receipt of the deposition transcript</p> <p>18 by you. If you fail to do so, the</p> <p>19 deposition transcript may be deemed to be</p> <p>20 accurate and may be used in court.</p> <p>21</p> <p>22</p> <p>23</p> <p>24</p>	<p>Page 429</p> <p>1 ACKNOWLEDGMENT OF DEPONENT</p> <p>2</p> <p>3</p> <p>4 I, _____, do</p> <p>5 hereby certify that I have read the</p> <p>6 foregoing pages, and that the same is</p> <p>7 a correct transcription of the answers</p> <p>8 given by me to the questions therein</p> <p>9 propounded, except for the corrections or</p> <p>10 changes in form or substance, if any,</p> <p>11 noted in the attached Errata Sheet.</p> <p>12</p> <p>13</p> <p>14</p> <p>15 _____</p> <p>16 MICHAEL BIRRER, M.D., PH.D. DATE</p> <p>17</p> <p>18 Subscribed and sworn</p> <p>19 to before me this</p> <p>20 _____ day of _____, 20____.</p> <p>21 My commission expires: _____</p> <p>22</p> <p>23 _____</p> <p>24 Notary Public</p>

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Exhibit 115



Review

The Role of Inflammation and Inflammatory Mediators in the Development, Progression, Metastasis, and Chemoresistance of Epithelial Ovarian Cancer

Sudha S. Savant ^{1,†}, Shruthi Sriramkumar ^{2,†} and Heather M. O'Hagan ^{1,3,*} 

¹ Medical Sciences, Indiana University School of Medicine, Bloomington, IN 47405, USA; ssavant@iu.edu

² Cell, Molecular and Cancer Biology Graduate Program, Indiana University, Bloomington, IN 47405, USA; ssriramk@iu.edu

³ Indiana University Melvin and Bren Simon Cancer Center, Indianapolis, IN 46202, USA

* Correspondence: hmohagan@indiana.edu; Tel.: +1-812-855-3035

† These authors contributed equally to this work.

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Abstract: Inflammation plays a role in the initiation and development of many types of cancers, including epithelial ovarian cancer (EOC) and high grade serous ovarian cancer (HGSC), a type of EOC. There are connections between EOC and both peritoneal and ovulation-induced inflammation. Additionally, EOCs have an inflammatory component that contributes to their progression. At sites of inflammation, epithelial cells are exposed to increased levels of inflammatory mediators such as reactive oxygen species, cytokines, prostaglandins, and growth factors that contribute to increased cell division, and genetic and epigenetic changes. These exposure-induced changes promote excessive cell proliferation, increased survival, malignant transformation, and cancer development. Furthermore, the pro-inflammatory tumor microenvironment environment (TME) contributes to EOC metastasis and chemoresistance. In this review we will discuss the roles inflammation and inflammatory mediators play in the development, progression, metastasis, and chemoresistance of EOC.

Keywords: inflammation; epithelial ovarian cancer; cytokines; reactive oxygen species; growth factors

1. Inflammation and EOC

Inflammation is part of the immune response that protects against foreign pathogens and aids in healing. Inflammation is elicited in response to cellular damage either by infection, exposure to foreign particles (pollutants or irritants), or an increase in cellular stress [1]. The ultimate goal of the inflammatory response is to restore tissue homeostasis, either by destruction or healing of the damaged tissue. The acute or immediate inflammatory response involves modification of the vasculature surrounding the site of stress or damage to increase blood flow. This alteration is then followed by activation of innate immune cells already present in the tissue, including macrophages, dendritic cells (DC), and mast cells, and an increase in infiltration of additional innate immune cells into the affected tissue. At sites of inflammation there are high levels of reactive oxygen species (ROS), cytokines, chemokines, and growth factors that are produced by the immune cells and other cells in the tissue. Acute inflammation is essential for tissue homeostasis and to protect against normal exposure to pathogens. However, in certain cases the body is unable to resolve this response or is subjected to repeated stimulation resulting in chronic inflammation.

Ovarian cancer (OC) is the fifth leading cause of cancer-related deaths in women in the United States [2] and can originate in the germ cells, sex-cord stroma, the fallopian tube (FT), or ovary

epithelium. Epithelial ovarian cancer (EOC) which originates from the ovary or fallopian tube epithelium, accounts for 85–90% of all OCs. Chronic inflammation is an important risk factor associated for EOC and high grade serous ovarian cancer (HGSC), the most malignant subtype of EOC. Chronic inflammation results in activation of signaling pathways, transcription factors, and the innate and adaptive immune responses [3,4]. In this review we primarily focus on inflammation as a risk factor for invasive EOC, but have also included supportive evidence from other OC subtypes, studies that do not define the subtype of OC, and other tumor types as indicated.

1.1. Signaling Pathways and Transcription Factors

Several signaling pathways and transcription factors involved in the inflammatory response also play critical roles in EOC. Here we briefly introduce relevant pathways that will be linked to OC formation in later sections. Cytokines produced during inflammation bind to and activate toll like receptors (TLRs) on cell surfaces, which results in activation of the signaling pathways involving mitogen-activated protein kinases (MAPKs) p38 and JNK (c-Jun N-terminal kinase) and transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the signal transducer and activator of transcription (STATs). The MAPK pathway regulates cellular processes like proliferation, differentiation, growth, migration, and cell death by upregulating the expression of transcription factors like AP-1, c-Jun, FOS and by activating NF- κ B and STATs, that either by themselves or along with AP-1 or c-Jun regulate expression of pro-survival and pro-growth genes. NF- κ B and AP-1 also regulate production of cytokines like IL-6 [5–7].

During inflammation these transcription factors play an important role to maintain tissue homeostasis. However, in case of chronic inflammation, the signaling pathways are continuously stimulated, which can contribute to tumorigenesis.

1.2. Innate Immune Response

Inflammation activates the innate immune response, which signals macrophages and DCs to secrete chemoattractants like Interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), and various other inflammatory mediators. These chemoattractants in turn result in recruitment of neutrophils, lymphocytes, and natural killer (NK) cells to the site of damage. All of these cells then secrete cytokines like IL-1, IL-3, IL-6, IL-8, tumor necrosis factor alpha (TNF- α), interferon (IFN) α , and colony-stimulating factors (CSF) like granulocyte macrophage CSF (GM-CSF). The cytokines bind to transmembrane receptors on the cell surfaces of other cells to activate transcription factors that regulate gene expression downstream of the cytokine activated pathway. This creates a pro-inflammatory environment resulting in recruitment of other immune cells, migration of endothelial cells, and proliferation of fibroblasts. Activation of macrophages and NK cells results in the production of high levels of ROS and reactive nitrogen species (RNS), which are used by these cells to kill foreign pathogens, but also end up damaging neighboring normal cells [8]. The lymphocytes also secrete growth factors like platelet derived growth factor (PDGF), transforming growth factor beta (TGF- β), and fibroblast growth factor (FGF), which facilitate wound healing. Overall the acute immune response is a rapid response that typically only lasts a few days. It results in removal of the pathogen, release of proteolytic enzymes to destroy damaged tissue, or stimulation of the proliferation of fibroblasts and epithelial cells to repair the tissue [1].

1.3. Adaptive Immune Response

If the infection is not resolved by the innate immune response, the adaptive immune response is activated, which is less inflammatory in nature. The adaptive immune response also provides longstanding protection against specific pathogens and/or antigens. B cells and T cells are the effector cells of the adaptive immune system that are derived from lymphocytes when they are presented with specific antigens by the antigen presenting cells (APC). T cells respond to the APCs by producing IL-2, which induces expression of transcription factors that facilitate T cells to differentiate

into T regulatory (Tregs) and T effector (Teff) cells. There are two major classes of T effector cells; CD8⁺ cytotoxic T cells and CD4⁺ T helper (Th) cells. Th cells are further differentiated into Th1, Th2, or Th17 depending on the ILs secreted and the transcription factors expressed. IFN- γ activates STAT1 to induce formation of Th1 and IL-6, and TGF- β can induce Th17 cell formation. Th1 and Th17 secrete ILs and activate macrophages and B cells to create a pro-inflammatory microenvironment (ME) that can be protumorigenic depending on the context. Tregs are immunosuppressive cells that turn off the immune response [1,9,10].

2. Inflammation as a Risk Factor for EOC

Amongst other factors such as hereditary, environmental, and lifestyle, inflammation emerges as an important risk factor for EOC. EOC arises either in the epithelial layer surrounding the ovary or in the epithelium of the distal FT, which could then spread to the ovary. A significant portion of HGSC is thought to originate in the FT, in part because removal of the FT significantly reduces OC risk [11]. Interestingly, while surgical specimens from mutation carriers rarely had premalignant ovarian epithelial changes, early lesions called serous tubal intraepithelial carcinomas (STICs) were found in the FTs of 5–10% of the patients. Copy number and mutational analysis suggest that STICs shed cells with metastatic potential that then colonize the ovary to form HGSC. STICs are mostly found in the fimbriae, the distal end of the FT that shares a ME with the ovary. During a woman's lifetime, the repeated secretion of ROS, cytokines, and other growth factors by the ovaries and immune cells creates a chronic inflammatory ME in the peritoneum that in turn potentiates the initiation of normal cells to malignant ones in the FT and the ovary, supports tumor progression, metastasis, and development of resistance to chemotherapy.

During ovulation, infection and other causes of inflammation ovary and FT tissue is damaged and undergoes repair. We will briefly discuss how each of these processes evoke or involve an inflammatory response that can persist, leading to a cytokine and growth factor rich environment in the peritoneum and contribute to EOC.

2.1. Ovulation

The process of ovulation itself is comparable to that of inflammation as described in the early 20th century. The development of the follicle to its rupture and release of the egg results in recruitment of activated immune cells to the ovary and production of enormous amounts of chemokines, cytokines, and growth factors. Ovulation is initiated by a surge of Luteinizing hormones (LH) that results in increased blood flow to the ovarian follicles. Before release of the egg, the surge of LH hormone recruits neutrophils and macrophages to the graafian follicles [12–14]. Macrophages in the theca have been shown to support growth of follicles [15]. During ovulation macrophages secrete growth factors like hepatocyte growth factor (HGF), TGF- β , and epidermal growth factor (EGF), which stimulate cellular proliferation and follicle growth. Simultaneously the macrophages also secrete ROS, TNF- α , and IL1 β , which stimulate local apoptosis resulting in rupture of the follicle, which bathes the ovarian surface and fimbriae with follicular fluid. Exposure of FT cells to follicular fluid results in altered expression of genes associated with inflammation, including increased expression of IL8 and cyclooxygenase-2 (COX-2) [16]. Quiescent fibroblasts are present in the thecal layer surrounding the follicles. Exposure to growth factors stimulates their proliferation and they then secrete prostaglandins, collagenases, and plasminogen activator. In the corpus luteum, after the follicle is released, the macrophages secrete prostaglandins, ROS, and TNF- α , which stimulate apoptosis of the corpus luteum cells. Therefore, ovulation results in the cyclic exposure of FT and ovarian epithelial cells to high levels of ROS, cytokines, and growth factors [17]. Although the other causes of inflammation discussed below are important and result in increased overall risk for EOC, the process of ovulation itself occurs often in the lifetime of the majority of women and may be the most important inflammation-related risk factor for EOC. This hypothesis is corroborated by the laying hen model, which is commonly used to study ovarian cancer [18]. In this model, hens develop spontaneous EOC, likely due to their high ovulation

rate, thus linking ovulation directly as an increased risk factor for EOC. Delayed onset of menarche and early onset of menopause have been shown to be inversely related to the risk of OC, likely due to the reduction in number of ovulation cycles in a woman's lifetime [19,20]. Further, ovulation has also been connected to EOC because contraceptive pills, pregnancy, and breastfeeding reduce the risk of OC. These factors reduce, halt, or delay overall ovulation cycles, respectively, which in turn reduces overall exposure to inflammation of the ovary and FT. The associations of parity and oral contraceptive use with invasive EOC were recently confirmed in a large, prospective study using the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort that found only limited heterogeneity in the risk between reproductive factors and EOC subtypes [21]. Hysterectomy, tube ligation, and removal of ovaries are also protective against development of OC [22,23].

2.2. Infection

Pelvic inflammatory disorder (PID) is the infection of the female reproductive organs like cervix, uterus, FTs, and ovaries. It is a significant risk factor for OC and is caused by various bacteria and virus such as *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Neisseria gonorrhoeae*, human papilloma virus, and cytomegalovirus [24,25]. Infection by these microbes results in DNA damage and production of ROS and induces a pro-inflammatory response, which involves secretion of cytokines and migration of immune cells [24]. PID is generally resolved with antibiotics within 48–72 hours of detection. However, repeated infection and unresolved inflammation can lead to chronic inflammation that is a risk factor for EOC.

2.3. Other Sources of Inflammation

The other causes of inflammation in the ovaries and/or FTs are endometriosis, obesity, Polycystic Ovarian Syndrome (PCOS), and talc exposure. Endometriosis is defined as presence of stroma and endometrial gland tissues in the pelvic peritoneum, rectovaginal septum, and ovaries [26]. Retrograde menstruation is the most commonly accepted theory for endometriosis. Retrograde menstruation results in aberrant accumulation of red blood cells (RBCs) and tissue, which can trigger an inflammatory response, activating the macrophages in the peritoneal cavity [27,28]. The macrophages lyse the RBCs, resulting in an increase in iron accumulation in the endometrial implants and peritoneal fluid. The accumulated iron can catalyze formation of free radicals like RNS and ROS in the peritoneum and results in increased oxidative stress (OS). OS can activate NF- κ B, in macrophages resulting in secretion of growth factors, cytokines, and IFNs. Around one third of women are affected by mild endometriosis, which resolves on its own over time. For the remaining cases, endometriosis results in chronic pain and inflammation, which can be resolved by excision of affected tissue or the outgrowth. However, in 45% of these cases, the endometriosis reoccurs resulting in repeated bouts of chronic inflammation [29,30].

Obese women have higher risks of EOC and HGSC and pro-inflammatory cytokines are associated with higher body mass index (BMI) levels. Adipose tissues secrete the cytokines TNF- α , IL-6, IL-8, and MCP-1, which can induce an inflammatory reaction in the peritoneum [31]. Continuous secretion of these cytokines leads to a state of chronic inflammation, which includes activation of macrophages and recruitment of NK cells and results in high levels of OS. Once the tumor has been initiated, the continuous secretion of cytokines by adipose tissue or omentum can facilitate migration of cancer cells to the omentum, promoting metastasis of the tumor into the peritoneum [30]. High levels (>10 mg/L) of C-reactive protein (CRP), a marker of global inflammation, are associated with an increased risk of EOC [32,33]. IL-6 itself is not a risk factor for EOC but in obese women IL-6 and CRP may be associated with increased EOC risk [33].

PCOS also contributes to inflammation in women and may increase risk of EOC [34]. PCOS is a hormonal disorder occurring in reproductive aged women during which ovaries may develop numerous small collections of fluid and fail to release eggs properly. Obesity, hyperandrogenism, and increased insulin resistance further characterize PCOS. Increased C-Reactive protein (CRP) and

MCP-1 levels, indicative of low-level chronic inflammation, are elevated in women with PCOS [35–38]. Simultaneously chemokines like IL-18, IL-6, and TNF- α are also increased in circulation in women with PCOS [39–42]. The increase in inflammatory mediators correlates positively with BMI, suggesting that increased obesity in women with PCOS may be the source of inflammation. Increased DNA damage and OS is observed in women with PCOS, which may also increase risk for EOC [43]. Evidence linking PCOS directly to EOC is limited due to small study sizes, PCOS being associated with other EOC risk factors such as obesity, and PCOS possibly being only associated with one subtype of EOC, borderline serous [44].

Talc is a silicate mineral and exposure to it can cause inflammation of the ovaries and poses a risk hazard for development of EOC [45]. It has been proposed that talc from talcum powder used for dusting and from condoms and vaginal diaphragms can migrate up to the ovaries via retrograde flow of fluids and mucous and get lodged in the ovaries. Tubal ligation, which is protective for EOC, is thought to block the transport of talc from the lower genital tract. Talc behaves as a foreign particle, triggering an inflammatory response [46,47]. The talc attracts macrophages, which try to phagocytose it. The macrophages then send chemotactic signals to other immune response mediators and initiate a wound healing process. Since talc is not degradable by the body, it inhibits the wound healing process, resulting in chronic inflammation.

2.4. NSAIDs and Reduced Risk of EOC

Further connecting inflammation to EOC are several studies that demonstrate that intake of non-steroidal anti-inflammatory drugs (NSAIDs), specifically of aspirin, correlates inversely with risk of OC and endometrial cancer [48–52]. In vitro studies with OC cell lines and NSAIDs show that NSAIDs and COX-2 inhibitors facilitate apoptosis, however this effect is not dependent on COX-2 and may be due to upregulation of p21, a protein important for cell cycle arrest [53]. Another study by Arango et al., demonstrates that acetylsalicylic acid or aspirin resulted in increased apoptosis via downregulation of Bcl2 in an endometrial cancer cell line [54]. A third study has shown that a selective COX-2 inhibitor, JTE-522, can inhibit proliferation and increase apoptosis of endometrial cancer cells by increasing levels of p53 and p21 and decreasing phosphorylation of retinoblastoma (Rb) protein, which results in its activation; all of which results in cell cycle arrest [55,56]. Simultaneously, there was an increase in caspase-3 activity, which is indicative of increased apoptosis. Another mechanism by which aspirin could facilitate its chemopreventive nature is by inhibiting oxidative induced DNA damage [57]. COX-1 is also expressed in normal ovaries of the laying hen, with expression increasing in post-ovulatory follicles suggesting its importance for or a role in ovulation. With the onset of OC, COX-1 expression is increased [58] and COX-1 inhibition and NSAIDs have shown to decrease proliferation of ascites in the laying hen OC model [59]. Further, when 0.1% aspirin was included in their diet for one year, although the onset of OC was not different, the progression of cancer was slower when compared to hens fed with regular diet [60].

As discussed, inflammation results in secretion of ROS, growth factors, cytokines, and chemokines into the shared environment surrounding the ovary and distal FT. Exposure of normal tissue to these inflammatory mediators results in activation of downstream signaling that can promote the transformation of normal cells or survival of already transformed cells. Once EOC has already formed further exposure of cancer cells to these inflammatory mediators also results in activation of downstream signaling within the cancer cell and in the surrounding tissue, creating an inflammatory environment that can further promote EOC (Figure 1). We will discuss in more detail how key inflammatory mediators contribute to EOC initiation, progression, metastasis, and chemoresistance.

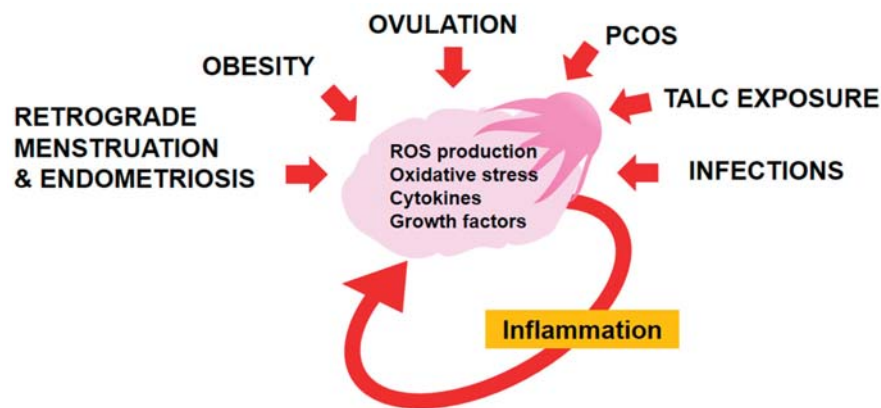


Figure 1. Sources of inflammation in the ovary and fimbriae. Ovulation, retrograde menstruation, endometriosis, infections, exposure to talc, Polycystic Ovarian Syndrome (PCOS), and obesity result in exposure of the ovary and fimbriae to reactive oxygen species (ROS), oxidative stress, cytokines, and growth factors, generating an inflammatory response that leads to additional production of ROS and cytokines in the ovary. Unresolved, chronic inflammation is a critical risk factor for tumor initiation.

3. Inflammation and EOC Initiation and Progression

Tumorigenesis is a multistep process that requires cells to gain the ability to evade apoptosis and antigrowth signals, proliferate independently of stimuli, develop a support system (angiogenesis), and have the capacity to invade and metastasize. Tumorigenesis is initiated by the transformation of a normal cell to a malignant one. The deregulation of the above mentioned processes in the malignant cell could potentiate its progression to cancer.

One mechanism of cancer initiation is genomic instability due to DNA damage [61] and EOCs exhibit a high number of chromosomal aberrations and genomic instability [62]. The most common gene mutations in HGSCs include *BRCA*, *TP53*, and genes involved in mismatch repair and the DNA damage response [63]. A pro-inflammatory ME can also contribute to genetic instability and therefore play a role in EOC cancer initiation. A pro-inflammatory ME, which is continuously supplemented by ROS, cytokines, and growth factors, can cause DNA damage in epithelial ovarian and FT cells, switch on antiapoptotic pathways, and initiate transformation of normal cells. When cells transformed either by oncogenic alterations or by exposure to inflammation are in a pro-inflammatory ME they are able to turn on pro-survival signaling pathways rather than the senescence pathways that are normally induced by oncogene expression in normal cells. For example, disruption of the RAS pathway results in activated NF- κ B signaling and upregulation of its downstream targets including cytokines like IL-1 β , IL-6, and IL-8. These cytokines are upregulated in EOC patients and their increased levels correlate with decreased survival [64–71]. The inflammatory mediators like cytokines, chemokines, growth factors, and prostaglandins secreted by the transformed epithelial cells further promote a pro-inflammatory environment, which can reprogram the surrounding cells to form the TME. The TME is mainly composed of endothelial cells, cancer associated fibroblasts (CAFs), adipocytes, tumor associated macrophages (TAMs), regulatory T-cells, pericytes, infiltrated immune cells such as neutrophils, lymphocytes, and various other cells that further secrete growth factors and cytokines which potentiate tumor progression (Figure 2, Table 1). Furthermore, OC-initiating cells (OCICs) have been identified in tumors and ascites that exhibit stem cell like properties and are capable of forming tumors [65,66,72]. Cytokines can promote self-renewal of CD133⁺ OCICs to potentiate tumor progression [73].

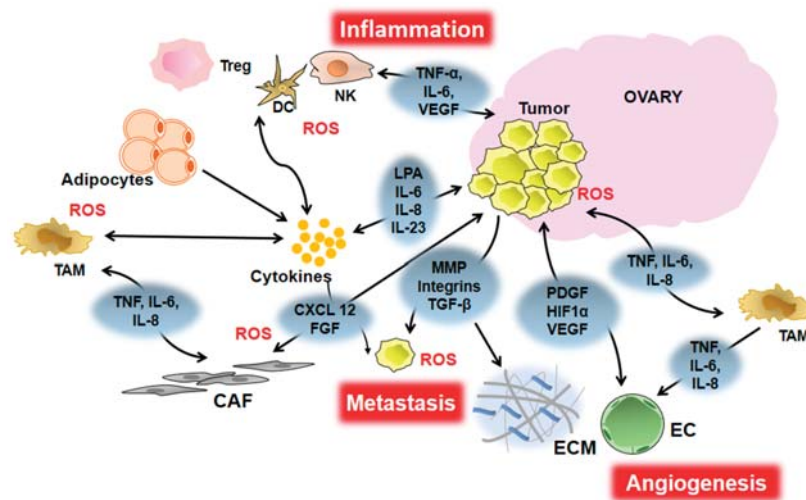


Figure 2. Inflammatory mediators contributing to EOC progression, metastasis, and angiogenesis. EOC cells produce ROS, chemokines, cytokines, and growth factors that can: (1) Lead to recruitment of immune cells like Dendritic cells (DC), Natural killer cells (NK), Tumor associated macrophages (TAMs), and T-regulatory (Treg) cells into the TME, which generate additional cytokines, ROS, and growth factors, resulting in chronic inflammation. (2) Stimulate the tumor cells themselves, the TAMs, and the surrounding fibroblasts (also known as cancer associated fibroblasts or CAFs) to proliferate and secrete growth factors like TGF- β and FGF that stimulate production of integrins and Matrix Metalloproteins (MMPs), resulting in migration of the tumor cell via degradation of the extra cellular matrix (ECM). (3) Stimulate endothelial cells (EC) to produce growth factors like PDGF and EGF and factors like VEGF that stimulate angiogenesis. The double arrows indicate that the cells are a source of the factor as well as stimulated by it.

The innate immune response can prevent tumorigenesis by recognizing and eliminating transformed cells. However, chronic inflammation can contribute to the ability of premalignant cells to evade apoptosis, escape the immune surveillance, and continue to grow, resulting in tumor formation. As mentioned, EOC can originate from either distal FT or ovarian epithelial cells. Since both the ovary and fimbria are exposed to the same ME, exposures reviewed here are relevant to initiation in either tissue. [74]. In this section we will review the role of OS and some specific pro-inflammatory mediators and signaling pathways in the initiation and progression of EOC.

3.1. ROS and Oxidative Stress

ROS plays an important role in the normal female reproductive cycle, from affecting maturation of the oocyte to ovulation, apoptosis of cells in corpus luteum, and embryo development [75]. Ovulation results in increased levels of DNA damage in the FT epithelium that is likely a result of the ROS generated during ovulation or the ovulation-associated increase in numbers of infiltrating macrophages in the FT [17]. Additionally, during infection and inflammatory responses immune and damaged cells produce ROS resulting in continuous exposure of the ovaries, FTs, and peritoneal cavity to ROS [76–78]. ROS exposure could potentially lead to epithelial cells in the ovary and FT undergoing transformative changes, as has been demonstrated for ovarian surface epithelium cells grown in 3D culture [79]. Elevated ROS and RNS levels beyond the level that cells can neutralize results in OS. Increased OS results in DNA damage, activation of signaling cascades, and epigenetic alterations.

DNA damage in a cell results in stimulation of DNA damage repair pathways. These repair pathways can be inactivated or be erroneous, which results in increased genotoxic stress and mutated DNA. Secretory tubal epithelial cells in the FT, a cell of origin for HGSC, are particularly susceptible to genotoxic injury with persistent DNA damage that could lead to mutation and STIC formation [80].

Mutations in tumor oncogenes and suppressors result in overexpression, constitutive activation of the protein, loss of expression, or expression of nonfunctional proteins, resulting in a transformed cell. Follicular fluid may have transformative properties as it has been demonstrated that bathing fimbriae with follicular fluid containing high levels of ROS results in increased levels of DNA damage. Bathing fimbriae that have loss of p53 and Rb with this follicular fluid results in evasion of apoptosis and cells with persistent DNA damage [81].

ROS can activate pro-survival intracellular tyrosine phosphorylation signaling cascades, mainly regulated by the MAPKs and redox sensitive kinases. Activation of c-Jun, JNK, ERK (extracellular signal-regulated kinase), and p38-MAPK signaling cascades results in upregulation of cell cycle proteins that enhances proliferation. Activation of JNK can also activate NF- κ B, which can suppress apoptosis. The MAPK pathway inhibits apoptosis and regulates differentiation. When activated in transformed cells these pathways are important for tumor initiation. ROS affects redox sensitive factors like thioreoxin, which is also found elevated in OC cell lines [82]. Thioredoxin is involved in redox regulation of transcription factors such as NF- κ B, NRF2, forkhead box class O (FOXO) proteins, reducing factor-1 (ref-1), and hypoxia inducible factor (HIF-1 α), thereby increasing their binding to the DNA. Most of these transcription factors promote tumor growth and progression by regulating expression of genes that affect cell survival and growth [83,84]. For example, FOXO, NRF2, and ref-1 transcription factors upregulate transcription of anti-oxidant proteins that scavenge free radicals and allow survival of damaged or transformed cells [85]. HIF-1 α upregulates the antiapoptotic factor, bcl-2 as well as vascular endothelial growth factor (VEGF), a factor important for angiogenesis.

OS has also been shown to facilitate epigenetic mechanisms in many cancers, including EOC [86]. Innate immune-mediated inflammation drives epigenetic silencing of tumor suppressor genes (TSGs) [87]. At sites of inflammation high levels of OS result in oxidative DNA damage that is recognized by the mismatch repair proteins mutS homolog MSH2 and MSH6. MSH2 and MSH6 then recruit epigenetic silencing proteins, including DNA methyltransferase 1 (DNMT1) to the sites of damage [88]. In an in vivo model of inflammation-driven colon tumorigenesis this early recruitment to sites of oxidative DNA damage results in permanent methylation of TSGs in tumors that form at the sites of inflammation [89]. While such a mechanism has not been directly proven in EOC models, Sapoznik et al. have demonstrated that exposure to follicular fluid or inflammation can induce Activation-Induced Cytidine Deaminase (AIDS) in fallopian tube epithelial cells, which results in epigenetic and genetic changes, increase in DNA damage and genotoxic stress and may be a contributing factor to EOC [90].

3.2. *TNF- α*

The cytokine TNF- α plays an important role in the process of ovulation and in removal of damaged corpus luteum. TNF- α ligand and its receptors, TNFRI and TNFRII are upregulated in ovarian tumors compared to normal ovarian tissue and high levels of TNF- α are found in ascites from OC patients [91–93]. OC cells have also been shown to secrete high levels of TNF- α as compared to normal ovarian epithelial cells resulting in autocrine upregulation of TNF- α mRNA and in expression of other pro-inflammatory cytokines, chemokines, and angiogenic factors like IL-6, M-CSF, CXCL2, CCL2, and VEGF [93,94]. Kellie et al. have shown using mouse models that TNF- α stimulates IL-17 production via TNFRI resulting in myeloid cell recruitment to the ovarian TME and increased tumor growth [95]. TNF- α , also upregulates AIDS transcript levels which can contribute to genotoxic stress [90].

3.3. *IL-6*

The cytokine IL-6 has been associated with poor survival in OC and is emerging as a potential therapeutic target for EOC [67,68,96,97]. IL-6 is normally produced by ovarian epithelial and OC cells. Macrophage migration inhibitory factor (MIF), EGF, and Transglutaminase secreted by OC cells can stimulate IL-6 production via activation of NF- κ B [98–100]. IL-6 increases proliferation of OC cells by

facilitating their exit from G1 into S phase of the cell cycle and by activation of the MAPK-ERK-Akt (protein kinase B) growth promoting signaling pathway [101]. ERK activation can promote formation of ascites by increasing the migration of tumor cells [70]. IL-6 production by M2 macrophages present in ascites in later stages of EOC can also stimulate cancer cell proliferation via STAT3 activation [102]. High levels of IL-6 can result in immune suppression by downregulation of IL-2, which stimulates Teff cell production [103]. IL-6 also stimulates production of Metalloproteinases (MMPs) in OC cells, which increases their invasive properties and promotes tumorigenesis [101,104].

3.4. IL-8

IL-8 a member of C-X-C chemokine family is present in the preovulatory follicle [105] where it may play a role in increasing leukocyte infiltration [106]. It is also elevated in ovarian cysts and in OC patients compared to healthy controls [107,108]. IL-8 has been found to be present in significantly higher levels in the ascites of patients with OC in comparison to patients with benign gynecological disorders [109]. Increased IL-8 expression has been associated with poor prognosis in OC patients [107]. Treatment of EOC cells with IL-8 results in their increased proliferation, which is accompanied by an increase in cyclins B1 and D1 and is dependent on phosphorylation of Akt and ERK [110]. Cyclins B1 and D1 are important for cell cycle progression, and an increase in their expression leads to increased cell growth. On the other hand, two independent studies have demonstrated that IL-8 inhibits EOC growth by increasing neutrophil infiltration [111,112].

3.5. Lyophosphotidic Acid (LPA)

LPA is a phospholipid that binds to and activates the endothelial differentiation gene (Edg) family of receptors. LPA is present in ovarian follicular fluid and it stimulates IL-6 and IL-8 production in the corpus luteum [113,114]. OC cells have been shown to produce LPA, which functions like a growth factor [115–119]. Plasma and ascites of OC patients have elevated levels of LPA that contribute to OC progression via upregulation of COX-2 and MMP2 [115,120,121]. LPA can bind to LPA₂ receptor and induce expression of IL-6 and IL-8 via activation of NF- κ B and AP-1 in OC cell lines [122]. It can induce ROS dependent Akt and ERK phosphorylation and inhibition of LPA can increase apoptosis of EOC cells [123]. ERK phosphorylation can induce phosphorylation of HIF-1 α , which then can upregulate VEGF and promote tumorigenesis. Another group demonstrated that stimulating EOC cells with ether-linked LPA resulted in their increased proliferation and survival by increased synthesis of DNA and activation of Akt via PI3K, which contributes to tumor progression [124].

3.6. Prostaglandins and COX-1 and COX-2

Prostaglandins are secreted in the ovary, FT, and uterus. They are important for maturation of the oocyte and facilitate the movement of the FT so that the mature oocyte can move from the ovary to the uterus. In the uterus prostaglandins help regulate and maintain uterine blood flow. COX-1 and COX-2 are enzymes that catalyze the production of prostaglandins from arachidonic acid and are overexpressed in OC patients [22,125,126]. High COX levels positively correlate with increased cell proliferation, angiogenesis, and malignancy in ovarian tumors [126,127]. COX-1 and COX-2 are normally involved in the acute inflammatory response but can become dysregulated in chronic inflammatory or TMEs. Obermayer et al. have demonstrated that prostaglandins produced by COX-2 can stimulate production of CXCR4 and its ligand Stromal cell derived factor 1 (SDF1) CXCL12 in myeloid derived suppressor cells (MDSC), which stimulates them to migrate towards OC ascites [128]. MDSCs inhibit the proliferation and differentiation of T cells, resulting in overall immune suppression, which allows the tumor cells to escape immune surveillance and continue to grow. Genetically engineered mouse models of EOC; one harboring the *p53* and *Rb* deletion and other the *KRAS*^{G12D} mutation and *Pten* deletion, demonstrate increased COX-1 levels, thus suggestive that COX-1 could be used as a potential biomarker and therapeutic target for EOC [129]. Further when

COX-1 was inhibited in EOC cells, it led to reduction in prostacyclin (a type of prostaglandin) synthesis and reduced tumor growth by enhanced apoptosis [130].

4. Inflammation and EOC Angiogenesis

Angiogenesis is required for the growth of both primary and metastatic tumors [131]. The process of angiogenesis is a complex multi-step process reviewed previously [132]. It is regulated by a balance between pro-angiogenic and antiangiogenic factors. Hypoxic and ischemic areas are present at sites of inflammation and also in tumors mainly due to obstruction of local blood vessels, differences in pace of growth of blood vessels and growth of the tumor and/or infiltration of immune cells. Macrophages accumulate at hypoxic sites and alter their gene expression profiles in response to the hypoxic conditions. One of the important genes for angiogenesis that is upregulated by hypoxia is VEGF [133,134]. The rate-limiting step in angiogenesis is VEGF signaling in endothelial cells (ECs) [135]. VEGF functions via tyrosine kinase receptors VEGF-1 and VEGF-2 and promotes migration, survival, proliferation of ECs, and formation of new blood vessels [136–138]. Many of the inflammatory mediators discussed so far are also involved in promoting angiogenesis in EOC as detailed below (Figure 2, Table 1).

4.1. *TNF- α*

TNF- α creates a pro-inflammatory TME and has also been associated with promoting angiogenesis. It has been hypothesized that *TNF- α* induces the production of soluble factors that promote tumor angiogenesis. Culture supernatants from *TNF- α* expressing cells induce the growth of mouse lung endothelial cells in vitro while culture supernatants from *TNF- α* lacking cells do not exert the same effect [94]. In pituitary adenomas *TNF- α* is known to induce VEGF that in turn induces CXCL12 [139,140]. VEGF and CXCL12 synergistically induce angiogenesis in EOC [141]. Mice injected with OC cells lacking *TNF- α* have reduced vascular density in their tumors and reduced formation of blood vessels in the peritoneal deposits. These mice also did not have accumulation of ascetic fluid suggesting the importance of *TNF- α* in angiogenesis and EOC progression [94].

4.2. *IL-6*

In physiological conditions, *IL-6* is involved in angiogenesis in the ovary during the development of ovarian follicles [142]. *IL-6* induces the phosphorylation of STAT3 and MAPK in ovarian endothelial cells thereby enhancing their migratory ability, a key step in angiogenesis [143]. As explained before, OC cells also secrete increased amounts of *IL-6*. Some OC cells also secrete an alternative splice variant of *IL-6R α* , the soluble form s*IL-6R*, which consists of only the ectodomain of the transmembrane receptor. By a process called trans-signaling, the s*IL-6R-IL-6* complex initiates signaling in cells in the ME that do not express the transmembrane receptor facilitating angiogenesis [144].

4.3. *IL-8*

Several studies have clearly established the role of *IL-8* in promoting angiogenesis. Hu et al., demonstrated that *IL-8* plays a role in angiogenesis using a rat sponge model [145]. *IL-8* was also able to induce angiogenesis in the rat cornea, which is normally avascular [146]. As explained in the previous section, there are several sources of *IL-8* in ovarian TME. Overexpression of *IL-8* in A2780 (non-*IL-8* expressing) OC cells has been shown to increase the expression of VEGF, MMP-2, and MMP-9; while depletion of *IL-8* in SKOV3 (*IL-8* expressing) cells has been shown to reduce VEGF, MMP-2, and MMP-9 [110]. The process of angiogenesis involves degradation of extracellular matrix components and proliferation and migration of endothelial cells. MMPs are a family of endopeptidases that breakdown components of extracellular matrix and have been implicated in angiogenesis [147]. Because of the importance of VEGF and MMPs in angiogenesis these findings suggest that *IL-8* in the ovarian TME will promote the formation of new blood vessels in EOC. Targeting *IL-8* using mouse models reduces EOC growth and decreases angiogenesis [112].

Table 1. Role of inflammatory mediators in different stages of tumor progression.

Inflammatory Mediators	Secreting Cell Type	Stages in Tumor Progression		
		Initiation and Progression	Angiogenesis	Metastasis
TNF- α ligands, TNFRI, TNFRII	OC cells, infiltrating monocytes, macrophages	\uparrow autocrine production of TNF- α and IL-6, M-CSF, CXCL2, CCL2 [93,94] and AIDS mRNA level [90]	\uparrow VEGF, VEGF \uparrow CXCL12 and promotes angiogenesis [139–141]	\uparrow TGF- α secretion by stromal fibroblasts which promote peritoneal metastasis [148] Enhances migration of OC cells towards CXCL12 [149,150]
IL-6	Ovarian epithelial cells, OC cells, M2 macrophages, mesothelial cells, TAMs, ascites	\uparrow Proliferation by promoting G1 to S transition and MAPK-ERK-Akt activation and STAT3 activation [101,102] \downarrow IL-2, resulting in immune suppression [103]	Induces STAT3 and MAPK phosphorylation which enhances migration of endothelial cells [143] sIL-6R-IL-6 facilitates angiogenesis in cells lacking IL-6 receptor [144]	Stimulates production of MMPs in OCs which \uparrow invasion and migration [101,104] \uparrow IL-6 in ascites enhances invasion via JAK-STAT signaling [151]
IL-8	Pre-ovulatory follicles, OC cells, ascites	\uparrow Proliferation by \uparrow cyclin B1 and cyclin D1 via pAkt [110]	\uparrow Expression of VEGF, MMP-2, MMP-9 promoting angiogenesis [110]	Activates TAK1 / NF- κ B via CXCR2 [153]
LPA	Follicular fluid, corpus luteum, OC cells, ascites	\uparrow IL-6 and IL-8 via NF- κ B and AP-1 [113,114,122] \uparrow COX-2 AND MMP2 [115,120,121] \uparrow phosphorylation of Akt and ERK resulting in increased cell cycle [123,124]	\uparrow Expression of VEGF via Myc and Sp-1 [155]	\uparrow urokinase, which results in degradation of basement membrane protein to promote metastasis [156,157]
Prostaglandins, COX-1 and COX-2	Ovary, FT, uterus, MDSCs	\uparrow CXCR4 and SDF1 in MDSCs resulting in immune suppression [128]	\uparrow Bcl-2 and blood vessel formation [158,159]	\uparrow Bcl-2, thus inhibiting apoptosis in lung, colon, breast and prostate cancers [158,159]
TGF- β and EGF	OC cells, CAFs		TGF- β \uparrow VCAN, which activates NF- κ B and \uparrow MM-9 [160]	\uparrow EGF protects cells from cisplatin-induced apoptosis [161]. Inhibiting TGF- β sensitizes resistant cells [162]

\downarrow Caspase-3 cleavage and makes OC cells resistant to cisplatin and paclitaxel [152] \uparrow Expression of MDRI, GSTpi, Bcl-2, Bcl-xL, and XIAP [152]

Blocks TRAIL induced apoptosis to promote resistance [154]

4.4. LPA

In addition to playing a role in initiation, and progression, LPA has also been implicated in angiogenesis in OC. LPA has been shown to induce transcriptional activation of VEGF in EOC cell lines [163]. Transcriptional activation of VEGF primarily occurs through HIF-1 α under oxygen limiting conditions in Hep3B hepatocellular carcinoma cells [164]. LPA mediated induction of VEGF expression has been shown to be independent of HIF-1 α in EOC cell lines. Transition metal cobalt treatment also leads to stabilization of HIF1 α similar to hypoxia. Combination treatment of EOC cells with cobalt and LPA additively increased VEGF production suggesting the effect of two different pathways [155]. LPA activates c-Myc and Sp-1, which induce VEGF expression through consensus binding sites in the VEGF promoter that have been implicated in HIF α independent induction of VEGF [155].

5. Inflammation and EOC Metastasis

Tumor metastasis is the major cause of mortality in most cancers, including EOC. Most EOC patients are diagnosed at an advanced stage when the cancer has already metastasized [165]. Dissemination of cancer cells to distant sites is a complex multi-step process called the invasion-metastasis cascade and is reviewed in detail in previous papers [166–168]. Briefly, some major steps in metastasis are—invasion through the basement membrane, intravasation into the lymphatics and circulation, survival of disseminating cancer cells in circulation, extravasation into surrounding tissues, colonization, and finally, formation of micro and macro metastases. However, unlike other epithelial malignancies, EOC has a different pattern of metastasis. EOC cells directly shed from the primary tumor into the peritoneal space and disseminate to organs in the peritoneal cavity. One of the prerequisites for cancer cells to metastasize is to undergo a process called epithelial to mesenchymal transition (EMT) where they lose their ability to attach to the basement membrane and acquire a mesenchymal phenotype and characteristics. Several recent evidences have indicated that the TME aids tumor cells to acquire these properties facilitating the metastatic cascade. An example of the ME promoting metastasis is the presence of STICs in the distal part of the FT, which shares its ME with ovary. Yang-Hartwich et al. have demonstrated that granulosa cells in the ovary secrete SDF-1 (stromal cell-derived factor 1) [169]. SDF-1 functions as a chemoattractant and recruits malignant FT cells to the ovary suggesting that the ovary is a primary site of metastasis, not the primary tumor site. Russo et al. demonstrated that loss of PTEN (phosphatase and tensin homolog) by the malignant FT cells and upregulation of WNT4 (wingless-related MMTV integration site 4) is crucial for initial metastasis to the ovary thereby supporting the tubal origin of EOC and the ovary as the primary site of metastasis [170]. The cells that make up the TME also secrete various inflammatory mediators, which facilitate progression and metastasis of OC cells (Figure 2, Table 1). These factors enable tumor metastasis by deregulating signal transduction pathways. Examples include the PI3-Akt and RAS-ERK pathways, which control migration and invasion through downstream effectors like Rho family GTPases, extracellular proteases, integrins, matrix associated proteins like focal adhesion kinases (FAK), and transcription factors like ETS2 and AP-1 [171–173]. Robinson-Smith et al. demonstrated that peritoneal inflammation correlated with dissemination of cancer cells from the ovaries in SCID mice. Augmenting the inflammatory response using thioglycolate accelerated ascites formation and metastasis while suppressing the inflammation using acetyl salicylic acid impeded ascites formation and reduced metastasis. This inflammation-induced metastasis of OC cells was found to be primarily mediated by macrophages and not neutrophils or NK cells [174]. As explained in one of the previous sections a pro-inflammatory environment can be created in the peritoneum due to secretion of cytokines like IL-6 and TNF- α by adipose cells [31]. Omentum, the primary site of metastasis of OC, is largely composed of adipose cells. In addition to adipocytes, omentum also consists of blood and lymph vessels, immune cells, and stromal cells [175]. Adipocytes have been shown to increase migration, invasion, and proliferation of EOC cells. Upregulation of SUSD2 a secreted tumor suppressor by adipocytes by guadecitabine treatment reduced EOC migration and invasion. This finding suggests that epigenetic changes in the stromal cells in addition to EOC cells can facilitate EOC

metastasis [176]. Omentum has aggregates of immune cells around the vasculature commonly referred to as milky spots [177]. Melanoma, lung carcinoma, ovarian carcinoma, and mammary carcinoma cell lines have been shown to specifically metastasize to the immune cell aggregates in the omentum when injected intraperitoneally into C57BL/6 mice [178]. These milky spots in the omentum have also been shown to facilitate metastatic colonization of the OC cells. Clark et al. have suggested that both adipocytes and milky spots have specific and important roles in metastatic colonization of OC cells [179]. These evidences imply that omentum potentially provides a good niche for the growth of ovarian cancer cells. Here we will specifically discuss how inflammatory mediators promote tumor metastasis in EOC.

5.1. ROS

EOC cells produce a large amount of ROS [180]. Loss of E-cadherin is one of the characteristic features of tumor cells with increased ability to migrate and invade. Wang et al. demonstrated that ROS leads to HIF α mediated activation of lysl oxidase. Lysl oxidase was shown to inversely correlate with E-cadherin expression promoting migration and invasion in EOC cells [181]. Tumor cells treated with sub-lethal doses of H₂O₂ failed to attach to the extracellular matrix components fibronectin and laminin and had increased metastatic colonization of lung, thereby establishing a role for ROS in tumor cell metastasis [182].

5.2. TNF- α

TNF- α provides a good example of how interactions between cancer and stroma aid in OC metastasis. Ascitic fluid and OCs contain a large number infiltrating macrophages in part because OCs constitutively produce M-CSF, which functions as a chemoattractant for monocytes [183]. These infiltrating monocytes produce many cytokines one of which is TNF- α [184,185]. OC cells also have elevated TNF- α expression that is regulated by DNA hypomethylation and chromatin remodeling of the TNF- α promoter. Increased TNF- α produced by OC cells and macrophages stimulates increased expression of TGF- α in stromal fibroblasts. TGF- α secreting stromal fibroblasts promote peritoneal metastasis of OC via EGF receptor signaling [148].

Furthermore, in EOC cells and clinical biopsies TNF- α expression correlates with one of the most commonly expressed cytokine receptors CXCR4. TNF- α stimulation of EOC cells enhanced their migration toward the only CXCR4 ligand, CXCL12. Stimulation of EOC cells by CXCL12 induced mRNA and protein expression of TNF- α . Therefore, a positive feedback loop has been suggested where in CXCL12 induced TNF- α potentially acts on the cancer cells and induces CXCR4 expression thereby enhancing tumor cell migration [149,150].

5.3. IL-6

IL-6 has also been implicated in metastasis of OC. Elevated levels of IL-6 found in serum and peritoneal fluid of EOC and OC patients have many sources [186–188]. Mesothelial cells in the peritoneum, TAMs, and EOC cells all secrete IL-6 [67]. M2 polarized macrophages in the ovarian TME induce proliferation and invasion of EOC cells by secretion of IL-6 [189]. Increased IL-6 present in ascites from OC patients enhanced the invasive ability of OC cells via the JAK-STAT signaling pathway. Canonically IL-6 signaling occurs by binding of the ligand to its transmembrane receptor IL-6R α . The effect of IL-6 on invasion of OC cells correlated with their IL-6R expression [151]. Because through trans-signaling, the sIL-6R–IL-6 complex initiates signaling in cells that do not express the transmembrane receptor [144], we hypothesize that IL-6 produced by macrophages could also promote invasion of OC cells similar to the mechanism of induction of angiogenesis.

5.4. IL-8

Increased proliferation, anchorage independent growth, and angiogenic potential are some prerequisites for cells to metastasize. IL-8 increases the proliferation of OC cells and upregulates VEGF

and MMP2 and 9 via activation of NF- κ B, which results in enhanced invasive phenotype of OC cells. IL-8 has been shown to activate TAK1/NF- κ B signaling via CXCR2, thereby facilitating the seeding and growth of OC cells in the peritoneal cavity during metastasis [153].

5.5. LPA

LPA promotes proliferation, survival, and metastasis of EOC cells by inducing the expression of c-Myc, VEGF, IL-8, MMPs and COX-2 [163,190–193]. LPA acts through its receptors LPAR1–3, which are members of G-protein coupled receptor superfamily. Invasive EOC cells have significantly higher expression of LPAR1 in comparison to non-invasive cell lines and LPA induces EOC cell invasion specifically through LPAR1 and not through LPAR2 or LPAR3 [194]. It can also induce secretion of urokinase in EOC cells, which has been shown to play a role in metastasis and its high levels correlate with advanced OC and poor survival in patients. LPA has been shown to increase promoter activity, mRNA levels, protein levels, and enzyme activity of Urokinase plasminogen activator (uPA) possibly via the edg-4 LPA receptor [156]. uPA is involved in converting plasminogen to plasmin, which facilitates the degradation of basement membrane and extracellular membrane proteins like fibronectin aiding in metastasis [157].

5.6. TGF- β

TGF- β initiates signaling by dimerization of serine/threonine kinase receptors. The dimerization of receptors results in their phosphorylation, which then relays signals downstream via SMAD dependent and SMAD independent pathways. Phosphorylation by the TGF- β receptor causes R-SMADs to bind to Co-SMAD and translocate to the nucleus, where they activate transcription of genes that promote invasion, migration. Bone morphogenic proteins (BMPs) are cytokines that belong to TGF- β family and have been associated with progression of many different cancer types. Their mechanism of promoting tumor progression depends on the TME in which the cancer grows and their mode of metastatic spread [195]. Specifically, BMP-2 overexpression has been associated with poor prognosis in OC [196]. Additionally, TGF- β could potentially modify the TME to promote tumorigenesis. Veriscan (VCAN), an extracellular matrix associated protein, was upregulated by TGF- β through TGF- β receptor II (TGFBR2) and SMAD signaling making the EOC cells more aggressive. Increased VCAN expression enhanced motility and invasion of EOC cells by activating NF- κ B signaling, increased expression of MMP-9, and hyaluronidase mediated motility receptor [160]. CAFs have higher expression of TGF- β receptors in comparison to normal ovarian fibroblasts and EOC cells suggesting that CAFs within the TME are more responsive to TGF- β than the other cell types [160].

6. Inflammation and EOC Chemoresistance

The standard treatment for EOC patients is cytoreductive surgery followed by platinum/taxane-based chemotherapy [197]. The main obstacle in treatment of EOC patients is development of chemoresistance. Resistance to chemotherapy can be either intrinsic or acquired. Inherent gene expression patterns harbored by chemo-naïve tumor cells contribute to intrinsic resistance. Acquired resistance is a consequence of different alterations induced after exposure to chemotherapeutic agents [198]. Different mechanisms, including increased drug efflux, decreased uptake of the drug, inactivation of the drug, increased DNA repair, and reduced apoptotic response, have been implicated in development of platinum resistance [199]. Several recent studies have demonstrated that the TME contributes to both intrinsic and acquired resistance. One type of intrinsic drug resistance influenced by the TME is referred to as environment mediated drug resistance (EMDR). In EMDR, factors and cells present in the TME activate diverse signaling events, transiently protecting the tumor cells from undergoing apoptosis in response to chemotherapeutic agents [200,201]. Another type of drug resistance induced by cytokines, chemokines, and growth factors secreted by fibroblast cells in the tumor stroma is called soluble factor mediated drug resistance (SFM-DR). A good example of SFM-DR is IL-6 mediated drug resistance in multiple myeloma. IL-6 is important for growth of multiple

myeloma cells. IL-6 activates STAT3 signaling in these cells and protects them from Fas mediated apoptosis by upregulating antiapoptotic protein Bcl-X_L [202]. Myeloma cells that produced IL-6 in an autocrine manner were found to be resistant to dexamethasone induced apoptosis while non-IL-6 producing cells were sensitive [203]. Cell adhesion mediated drug resistance (CAM-DR) occurs due to adhesion of tumor cells to extracellular matrix components like laminin, collagen, and fibronectin or due to fibroblasts present in the tumor stroma [204]. An example of this type of resistance is when drug sensitive myeloma cells were adhered to an extracellular matrix component fibronectin, they exhibited a reversible drug resistant phenotype which was not due reduced drug accumulation or increase in antiapoptotic proteins like Bcl-X_L [201]. Here we will discuss specific inflammatory mediators and their role in OC chemoresistance (Figure 3).

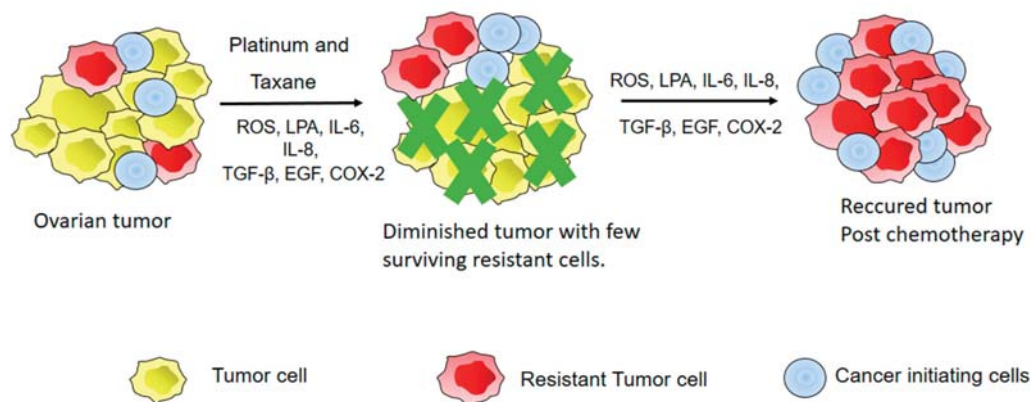


Figure 3. Inflammatory mediators contribute to chemoresistance of EOC. A combination of platinum and taxane drugs is currently used as chemotherapy for OC. ROS, Lyophosphotidic Acid (LPA), cytokines, and growth factors like TGF- β and EGF increase tumor cell survival by upregulating antiapoptotic genes, by stimulating stemness and proliferation of cancer initiating cells, by increasing repair of damaged DNA, or by increasing efflux of the drug. The resistant tumor cells and the cancer initiating cells can then proliferate under the influence of growth factors and cytokines resulting in a recurrent chemoresistant tumor.

6.1. ROS

ROS are abundant in the pro-inflammatory TME. Malignant EOC tissues have been shown to have 96% higher ROS levels than normal controls [205]. OC stem like cells or OCICs are more drug resistant and responsible for relapse of chemoresistant tumors [66]. OCICs produce ROS and superoxide. This ROS induces the expression of peroxisome proliferator-activated receptor-gamma coactivator (PCG)-1 α , which regulates mitochondrial biogenesis and is required for expression of detoxifying enzymes [206,207]. PCG1 α increases the aldehyde dehydrogenase (ALDH) activity and expression of multidrug resistance gene (MDR1). MDR1 is an ATP dependent transporter that has been associated with efflux of platinum based drugs from OC cells contributing to platinum resistance. Scavenging ROS reduced expression of PCG1 α and drug resistant related genes thereby linking ROS to development of chemoresistance [207].

6.2. IL-6

IL-6 in the OC TME is associated with increased chemoresistance. Wang et al. demonstrated that autocrine production of IL-6 by EOC cells makes them resistant to cisplatin and paclitaxel by causing decreased proteolytic cleavage of capase-3. Paclitaxel resistant EOC cells have increased expression of IL-6 and one of its downstream effectors STAT3 [208,209]. IL-6 producing OC cells also had increased expression of multidrug resistant genes MDR1 and GSTpi and anti-apoptotic genes

Bcl-2, Bcl-xL, and XIAP, suggesting that IL6 promotes drug resistance by increasing drug efflux and reducing apoptosis [152].

6.3. IL-8

IL-8 blocks TRAIL-induced apoptosis and reduces caspase cleavage in EOC cell lines by decreasing the expression of death receptor (DR) 4 [210]. TRAIL is a cell death inducing ligand that belongs to the TNF superfamily and has been shown to induce apoptosis specifically in tumor cells and not in nontransformed cells [211,212]. Combination of TRAIL and the chemotherapeutic drugs—cisplatin, doxorubicin, and paclitaxel has been shown to induce apoptosis in chemoresistant EOC cell lines by causing increased caspase and PARP cleavage [154]. This finding suggests that IL8 may contribute to chemoresistance by blocking TRAIL.

6.4. LPA

LPA has been shown to contribute to platinum resistance by preventing cells from undergoing cisplatin-induced apoptosis without affecting their proliferation rate. The mechanism of how LPA inhibits apoptosis in EOC cells in response to cisplatin is not yet clearly understood [161].

6.5. TGF- β and EGF

Recurrent OC show significantly higher expression of TGF- β 1 and TGF- β 3 in comparison to primary tumors and normal ovary tissue [213]. Inhibition of TGF- β by the inhibitor LY2109761 sensitizes resistant SKOV3 cells to cisplatin suggesting that TGF- β contributes to the development of platinum resistance in EOC cells [162]. Cisplatin resistant A2780P cells had hypomethylation and upregulation of TGFBR2 confirming the involvement of the pathway in acquisition of platinum resistance [214]. An elevated level of EGF receptor (EGFR) has also been associated with poor prognosis in OC patients [215]. EGF has been shown to stimulate the growth of EOC cells expressing EGFR and alters their cell cycle distribution [216]. EGF similar to LPA has been shown to protect EOC cells from undergoing cisplatin induced apoptosis [161].

6.6. COX-2

In addition to being associated with tumor initiation and progression, COX-2 has also been associated with chemoresistance. Ferrandina et al. reported that a statistically significant higher percentage of primary OC patients unresponsive to platinum-containing chemotherapy were positive for COX-2 than responsive patients (84.6% versus 34.6%, respectively) [217]. The percentage of positive COX-2 staining per tumor area in COX-2 positive patients ranged from 15 to 45%. The results from this study suggest that COX-2 levels may influence the response of patients to different chemotherapy regimens, but the sample size of this study was small and the results need to be confirmed in a larger group of patients. Furthermore, this association needs to be corroborated biochemically [217]. In both patients groups undergoing cytoreductive surgery and explorative laparotomy, COX-2 expression was higher in nonresponders [218]. Using lung, colon, and prostate cancer models, COX-2 has been shown to induce Bcl-2 and promote tumor growth by facilitating the formation of new blood vessels [158,159]. These findings suggest that COX-2 may contribute to chemoresistance by inhibiting apoptosis and promoting angiogenesis in OC as well.

7. Treatment Strategies Targeting Inflammatory Mediators in EOC

As discussed, development of resistance to available chemotherapeutic drugs remains the major obstacle in management of OC patients. While several immunotherapies have been developed to improve the antitumor response of T-cells and/or modulate the immune response, here we will discuss EOC treatment strategies that specifically target the inflammatory mediators that have been reviewed above.

A monoclonal antibody directed at VEGF, bevacizumab, has been widely studied and is a promising target in EOC [219]. Bevacizumab is a recombinant humanized monoclonal antibody and has been approved by the FDA for treatment of metastatic breast, non-small cell lung, and colorectal cancer. Phase II clinical studies have shown that it is active in treatment of recurrent OC patients [220]. OCEANS trial was a randomized phase III clinical trial that evaluated the safety and efficacy of bevacizumab in combination with gemcitabine and carboplatin (GC) in comparison with GC alone in recurrent platinum sensitive ovarian, primary peritoneal, or FT cancer. This trial demonstrated that bevacizumab was able to prolong the PFS in platinum-sensitive recurrent EOC patients [221]. In addition to OCEANS, GOG218, and ICON7 have also shown that bevacizumab prolongs the PFS in OC patients confirming the promise this therapeutic target holds for management of OC [222,223].

We have discussed some mechanisms by which the pro-inflammatory cytokine TNF- α promotes OC metastasis and angiogenesis making it a good target for development of therapeutic agents. The safety profile and biological activity of a monoclonal anti-TNF- α antibody, Infliximab was assessed in a clinical study consisting of patients with advanced solid tumors, including OC. Infliximab did not have any toxic effects and was well tolerated by these patients. Reduced plasma levels of IL-6 and CCL12 in these patients was observed 24 h and 48 h after administration of Infliximab, while neutralization of TNF- α was detected after an hour indicating some biological activity [224]. This response warrants further study of Infliximab as a therapeutic agent for treatment of OC.

IL-6/STAT3 signaling has been implicated at different stages of OC progression and is a promising target although most agents are still in preclinical or early clinical trial stages. Siltuximab, an anti-IL-6 antibody, suppresses IL-6-induced STAT3 phosphorylation and nuclear translocation in OC cell lines. Siltuximab treatment also reduced the level of pro-survival proteins like Bcl-X_L and Survivin, which are downstream of STAT3. Siltuximab was able to sensitize paclitaxel resistant OC cell lines, but did not show the same effect in vivo [225]. sc144 is a novel small molecule inhibitor has shown significant promise in preclinical studies. sc144 binds gp130, which is a signal transducer in STAT3 signaling. It causes phosphorylation of gp130 leading to its deglycosylation. This abrogates downstream STAT3 phosphorylation and nuclear translocation inhibiting transcription of downstream genes. sc144 has increased potency in EOC cells in comparison to normal epithelial cells and slows down the growth of tumors in xenograft models of EOC [226]. A phase I clinical trial combining carboplatin, the monoclonal antibody Tocilizumab, which blocks IL-6R, and immune enhancer INF- α showed good promise. The EOC patients who received the highest dose of Tocilizumab had increased serum levels of IL-6 and sIL-6R and also showed longer median overall survival [227].

We have discussed the role of TGF- β in EOC tumor progression substantiating it as a good therapeutic target. A preclinical study of LY2109761 (TGF β RI and TGF β RII kinase inhibitor) in combination with cisplatin was conducted by Gao et al. This inhibitor significantly increased apoptosis in cisplatin resistant cells. Combining LY2109761 with cisplatin had antiproliferative effects and increased the rate of apoptosis in parental and cisplatin resistant xenograft models [162]. In triple negative breast cancer, LY2157299 a TGF- β 1 receptor kinase inhibitor, prevented recurrence of tumors in xenograft models after treatment with paclitaxel [228]. Early phase clinical trials of LY2157299 in patients with advanced or metastasized pancreatic cancer have been completed. Early phase trials in triple negative metastatic breast cancer, unresectable hepatocellular carcinoma, and metastatic castration resistant prostate cancer are underway [229].

EGF has also been associated with chemoresistance in EOC. Cetuximab, a chimerized monoclonal antibody that targets EGFR, was tested in combination with carboplatin in patients with recurrent platinum sensitive OC. Cetuximab showed modest activity in these patients [230]. Panitumumab, a human monoclonal antibody specific to EGFR, in combination with carboplatin did not improve efficacy or progression free survival in platinum sensitive EOC patients [231].

8. Conclusions and Future Perspectives

Several studies in the last decade have associated increased inflammation and inflammatory mediators with increased EOC risk and reduced survival in EOC patients. We have presented published evidence suggesting that inflammation and inflammatory mediators promote ovarian tumorigenesis. However the mechanisms by which the process of inflammation culminates in ovarian tumor initiation need to be further understood. Such links have been established in colon and pancreatic cancer. Understanding these mechanisms is important for developing ways to target inflammatory mediators and reduce OC risk. Furthermore, epidemiological studies of NSAIDs and early clinical trials targeting IL-6 and TNF- α have shown significant promise, thus suggesting that targeting inflammatory mediators as treatment for OC warrants future research.

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